

EFFECT OF ZILPATEROL HYDROCHLORIDE AND STEROID IMPLANTATION ON
YEARLING STEER FEEDLOT PERFORMANCE, CARCASS CHARACTERISTICS, AND
SKELETAL MUSCLE GENE EXPRESSION

by

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Abstract

Zilpaterol hydrochloride (ZH) is a growth promotant that is approved for use in finishing cattle to improve growth performance and increase lean tissue accumulation. Little is known about the combined effects of ZH with anabolic steroid hormone implants. There is also little published data on the effect these growth promotants have on genes that play a role in skeletal muscle synthesis and degradation. Therefore, two separate studies were conducted to address these issues. The first study evaluated the effects of ZH and the steroid implant Revalor-S (RS) on animal performance and skeletal muscle gene expression in feedlot steers. Four treatments were used to analyze the effects of RS implanted 58 days before ZH, which was fed for 30 days with a 3 day withdrawal. It was determined that ZH and RS additively contribute to improved live and carcass performance; however these compounds had different effects on the abundance of the receptors for ZH as well as the abundance of myosin heavy chain (MHC) mRNA in skeletal muscle of feedlot steers. It was also determined that ZH can cause a transition in the abundance of MHC mRNA isoforms in skeletal muscle that are available for the translation of larger, faster, more glycolytic fiber types of MHC. The second study evaluated the effects of two types of anabolic steroid hormones on myosin heavy chain gene expression. Four treatments were used to measure the effects of trenbolone acetate (TBA) and estradiol (E₂) on performance and the amount of MHC mRNA in skeletal muscle of finishing steers. It was determined that anabolic steroid implants improve live animal performance, however there was no alteration in the abundance of MHC mRNA in skeletal muscle of feedlot steer for 28 days after implantation; however there was an increase in intermediate fiber type IIA of MHC mRNA in skeletal muscle with increasing days on feed. From these studies we concluded that ZH and anabolic steroids do have an effect on growth performance; however they may differ in the distinct mechanism of action utilized to enhance lean tissue deposition in feedlot steers.

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CHAPTER 1 - Review of Literature

Introduction

One of the major factors involved in profitability in the beef cattle industry is the cost associated with animal weight gain. Due to this, there have been advancements made in the production of exogenous compounds that can be administered to cattle to improve the efficiency with which an animal can convert energy consumed into body mass. Furthermore, these compounds have the capability to alter the composition of the weight accumulation within the animal. Special attention has been given to the lean tissue involved in this weight gain in the beef cattle industry. With current feed cost at record high levels, the cattle industry needs to utilize the FDA-approved technologies available for improved growth efficiency.

Currently, there are two classes of growth promoting agents that are approved for use in the cattle finishing industry that have been shown to stimulate lean tissue deposition. The most widely utilized growth promoters are anabolic steroids, which have been approved and widely used for over 50 years. The other type of growth promoting agent is phenethanolamines, also known as β -adrenergic agonists (β AA). These synthetic compounds have only been utilized with finishing cattle in the United States for the last 5 years, and they elicit similar biological responses as anabolic steroids, but this is achieved through pathways that appear to differ from those of anabolic steroids. This review will look at the effects of skeletal muscle development with different types of growth promoters, and how their combined use may affect performance and the composition of growth in finishing beef cattle.

Characteristics of Postnatal Skeletal Muscle

Skeletal muscle is a specialized tissue within mammalian species that has various functions, and it is of great interest in meat producing livestock systems. The structural components of muscle function within mammals to generate contractile energy as well as being involved in many other biological functions. A unique characteristic of skeletal muscle is that the primary cellular unit consists of individual muscle fibers. The numbers of these multinucleated muscle fibers, as well as the nuclei, are post-mitotic or fixed at birth. They do however have the post-natal capability to increase in size by means of hypertrophic growth. It has been shown that DNA synthesis precedes mitosis (Messier and LeBlond, 1960). Furthermore, hypertrophic growth is directly related to the DNA accumulation within the fiber that is necessary to increase

the size of the fiber by the transcription of DNA to RNA and subsequently the translation of protein (Trenkle et al., 1978). Because the number of nuclei present on each fiber is fixed, then the amount of DNA available for growth and protein synthesis is limited. However, during growth these fibers do increase in the amount of DNA accumulated. From 60 to 90% of the DNA found in the muscle fiber is accumulated during the growth of the animal, postnatally (Allen et al., 1979). From this confounding evidence, a specialized type of myogenic cell was discovered. Satellite cells are the source of this postnatal DNA accumulation.

Satellite Cells

Satellite cells are mononucleated cells that are located between the basal lamina and sarcolemma of muscle fibers (Mauro, 1961). Moss and LeBlond (1970) observed, by use of electron microscope, two distinct types of nuclei located on the muscle fiber. These nuclei had distinct physical features and differed in their location relative to the sarcolemma of the fiber. Moss and LeBlond (1970) conducted a study where mice were injected with ^3H -thymidine and sacrificed at intervals from 1 to 72 hours later. From radioautograph results, there was no incorporation of ^3H -thymidine in the true muscle nuclei 1 hour after injection, which suggests that they are not dividing. The satellite cells were labeled 1 hour after the injection, which suggests that they were synthesizing DNA and able to divide. Another interesting result they found was the number of labeled satellite cells decreased over time, and the number of labeled true muscle nuclei increased over time. From this it was determined that satellite cells can divide and incorporate into existing muscle fiber (Moss and LeBlond, 1970). However, once the satellite cell has incorporated into the fiber, they terminally lose their ability to proliferate (Moss and LeBlond, 1971). However, if all of the satellite cells were to incorporate and lose their ability to proliferate, hypertrophic growth would not be able to continue throughout the animal's life. In fact, one half of the daughter cells from satellite cells that had proliferated were able to incorporate into existing muscle fibers, and the other half of the non-incorporated daughter cells was able to continue proliferating (Cardasis and Cooper, 1975). These non-incorporated satellite cells are able to enter a state of quiescence or G_0 of the cell cycle (Cardasis and Copper, 1975). This process provides a reserve of satellite cells for injury repair as well as additional growth as an animal progresses toward maturity. This poses a challenge in optimizing skeletal muscle growth in meat producing animals as they progress toward maturity. However, the obligatory role of satellite cells in acute muscle hypertrophy has been an issue of dispute.

There is continued controversy over the hypothesis that hypertrophic growth can take place without incorporation of more satellite cell DNA into the existing muscle fiber. This hypothesis rests on the concept that the muscle fiber can increase the efficiency of the DNA already present to transcribe RNA and subsequently translate more protein. The DNA unit is the ultimate limiting factor for this process, therefore any hypertrophy independent of additional DNA will eventually reach a limit to the growth potential of the myofiber nuclei unless more DNA becomes available. This becomes a more pressing issue as an animal reaches a more mature age, because the number of satellite cells could be affected by age. Approximately 30% of the muscle nuclei are satellite cells in a newborn, however that amount drops as low as 2 to 10% in mature animals, which shows that the actual number of satellite cells decreases with age (Cardasis and Cooper, 1975). Furthermore, if exogenous compounds are capable of either promoting growth with or without satellite cell stimulation, research could be implemented to elucidate the extent of satellite cell's obligatory role in muscle development. Therefore any factors that affect satellite cells could be key elements in optimizing growth in cattle, especially near maturity.

Growth Factors

There are various transcription and growth factors that play a part in moderating the activity of satellite cells. The primary growth factor known to activate quiescent satellite cells is hepatic growth factor (HGF). It was determined that HGF is present in normal adult skeletal muscle and is capable of activating satellite cells (Tatsumi et al., 1998). A study by Allen et al. (1995) found that when HGF was exposed to satellite cells in culture, the lag time before quiescent cells enter the cell cycle was significantly shortened. It was also found that quiescent satellite cells express mRNA for c-met (Allen et al., 1995), which is the receptor for the ligand HGF (Bottaro et al., 1991). Another important determinant of satellite cell fate is the Notch signaling pathway. The proliferative expansion and myogenic lineage progression of quiescent satellite cells is regulated and maintained by the Notch signaling pathway (Conboy and Rando, 2002). The activation of Notch-1 ligand, Delta, is one of the first changes associated with satellite cell activation, followed by the up regulation of Numb, an antagonist of Notch-1. Increased Numb expression leads to the expression of myogenic regulatory factors, desmin and Pax-7, which are indicators of proliferation and commitment of satellite cells to a myoblast cell fate (Conboy and Rando, 2002).

Other factors affect the proliferation and differentiation of activated satellite cells. In an in vitro study conducted by Greene and Allen (1991), bovine satellite cells were cultured and exposed to basic (b) FGF, IGF-I, and TGF- β to evaluate their effect on satellite cells. When individually administered, the bFGF was found to stimulate proliferation, but inhibit differentiation. The IGF-I had no effect on proliferation, but stimulated differentiation, where as TGF- β inhibited both proliferation and differentiation. They further analyzed the effects on satellite cells when administered in combination. The maximum stimulation of proliferation occurred in the presence of bFGF and IGF-I, but there was no effect on differentiation. When bFGF was in the presence of TGF- β proliferation was stimulated, but differentiation was inhibited (Greene and Allen, 1991). One member of the TGF- β superfamily that is responsible for negative regulation of skeletal muscle is myostatin (McPherron et al., 1997). Embryonic mutations causing the inactivity of the myostatin gene lead to the condition known as double muscling in cattle (McPherron and Lee, 1997). This data suggests that these growth factors may play a critical role in regulating bovine satellite cell activity, and interaction of these growth factors may also play a role in that activity.

Myosin Heavy Chain Isoform

Myosin is the most abundant protein present in striated muscle cells, comprising approximately 25% of the total protein pool (review by Baldwin and Haddad, 2001). Myosin serves as the backbone of the sarcomeric structure. It is also an enzyme that utilizes ATP and generates movement by binding with another sarcomeric protein, actin. This chemical energy is the force that generates a muscle contraction and enables physical body motion. As functional demands on muscle change, muscle fibers have the capacity to change their overall metabolism and contractility in response to various stimuli (Pette and Staron, 2000). Certain conditions of altered neuromuscular activity, mechanical loading, and hypothyroidism can cause MHC isoforms to transition from fast-to-slow or slow-to-fast fiber types (Pette and Staron, 2000).

The four isoforms of MHC that are predominantly found in different proportions within adult skeletal muscles of various mammalian species are MHC types I (also referred to as β /slow), IIA, IIX, and IIB (Schiaffino and Reggiani, 1996). Differences in sequencing of the loop 1 and loop 2 regions of MHC isoforms have been found responsible for functional differences of MHC fibers (Chikuni et al., 2004). The loop 1 sequence is related to the rate of ADP release and affects ATPase activity (Sweeny et al, 1998; Chikuni et al., 2004). Comparing bovine and

porcine MHC, the major diversity in the loop 1 region was the differences between the fast and slow type isoforms (Weiss et al., 1999; Chikuni et al., 2002). The loop 2 region, however, has different functional aspect. The structure of the myosin head domain indicates that the loop 2 region acts as an actin-binding site (Rayment et al., 1993), and sequence substitutions on the loop 2 region can cause changes in ATPase activity (Ueda et al., 1994). Substitutions on the loop 2 region might tune the rate-limiting step in the actin-activated ATPase cycle (Spudich, 1994), and be a major determinant of V_{max} of the ATPase activity (Ueda et al., 1994). The sequence of the loop 2 region is different among all isoforms of MHC in cattle, as well as in humans and pigs (Weiss et al., 1999; Chikuni et al., 2001, 2002). These studies imply that the functional properties vary between fiber type and the bovine myosin can differ from porcine myosin within the same isoform or fiber type (Chikuni et al., 2004). An example of this the MHC type IIB isoform that is present in porcine muscle, but whose presence is questioned in cattle muscle. Recent findings have challenged the existence of type IIB MHC isoform in bovine skeletal muscle tissue, and have determined that no type IIB MHC mRNA could be detected in adult skeletal muscle RNA of cattle (Chikuni et al., 2004), however there is type IIB MHC fibers found only in specialized extraocular muscles in cattle (Toniolo et al., 2005).

In regard to growth of meat producing animals, performance has been related to muscle fiber type composition in pigs (Sosnicki, 1987). Data shows that rats selected for rapid postnatal weight gain have more fast-contracting fibers (Swatland and Cassens, 1972). Furthermore, muscle from animals with certain genetic anomalies such as; double-musced cattle, callipyge sheep, and halothane-positive pigs have been shown to have a greater proportion of fast, glycolytic muscle fibers (Lefaucheur and Gerrard, 2000). In contrast, domestic pig studies determined there was no evidence of differences in muscle fiber type composition due to selection for high lean growth (Karlsson et al., 1993; Larzul et al., 1997). Other studies further determined that induced muscle fiber hypertrophy observed with growth hormone had no effect on muscle fiber type (Beermann et al., 1987; Solomon et al., 1991). It is important to note, however, that many of these studies were not capable of utilizing fiber-typing protocols the could distinguish all four adult muscle fiber types, especially IIX and IIB, which represent approximately 80% of the total fibers in the longissimus muscle of pigs (Larzul et al., 1997). Therefore, changes in MHC isoforms could be used to detect alterations in muscle's functional characteristics and carcass muscle composition as a result of growth promoter administration in

cattle. Furthermore the same fiber type of MHC may differ in functional characteristics between species, and this may explain differences in types of isoforms detected between porcine and bovine species. Studies have also measured the relative amount of myosin light chain (MLC) isoforms and found that extensive variations exist in isoforms expressed in skeletal muscle fibers, yet there are distinct patterns among different species and among muscles within an individual specie (Bicer and Reiser, 2004). More recent studies have set out to measure changes in MHC fiber type as a result of exogenous growth promoter use to analyze alterations in skeletal muscle hypertrophy. These reports will be addressed later in this review.

Anabolic Steroids

Anabolic steroids belong to the steroid super-family and are potent stimulators of skeletal muscle growth. Steroidogenic enzymes are responsible for the biosynthesis from cholesterol of various steroid hormones including glucocorticoids, mineralcorticoids, progestins, androgens, and estrogens (Sanderson, 2006). The adrenal gland is an important steroidogenic tissue in the human body, and unlike the gonads, it produces adrenal hormones that are essential for survival (Addison, 1855). Steroid hormone ligands are highly hydrophobic in nature which allows them to pass through the plasma membrane (Geisler, 2003). The two primary classes of anabolic steroids currently used in the cattle finishing industry are androgens and estrogens. Both of these steroids have a biological effect in most livestock species as well as in humans. The purpose of anabolic steroid use in livestock and specifically cattle is to improve the rate and efficiency of gain. Trenbolone is a synthetic androgen that is similar in structure to testosterone (Anderson, 1991). Trenbolone acetate (TBA) has approximately 50 times the anabolic potency of testosterone and is 8-10 times stronger than testosterone propionate (Neumann, 1976). Estradiol-17 β (E₂) is an estrogenic hormone that is naturally produced in vivo, and can be synthetically produced. The combination of TBA and E₂ were approved for use in the United States in 1992, and are the most common combination of steroidal implants used currently in finishing cattle.

Effect of Anabolic Steroid on Growth

Cellular responses to steroid hormones are mediated by receptors that initiate a complex range of cellular events upon ligand binding. These cellular responses can be classified into the two broad groups of genomic or rapid signaling responses. The genomic response is the classical response that is characterized by changes in gene transcription and occurs over the time course of

hours to days (Prossnitz et al., 2008). With genomic responses, steroid ligands bind to the intracellular steroid receptors and effect the regulation of transcription factors and protein synthesis (Beato et al., 1996). There are two known estrogen receptors. The first one was discovered in 1973 and is termed ER α (Jensen et al., 1973). The second estrogen receptor was discovered in 1996 and is termed ER β (Kuiper et al., 1996). Testosterone action is mediated by the androgen receptor, which transduces the steroid signal within cells (Mateescu and Thonney, 2005). It is thought that TBA may act on skeletal muscle through glucocorticoid receptors to reduce the catabolic effect of glucocorticoids (Muir, 1985). It has also been suggested that testosterone can up-regulate RNA polymerase and ribosomal activity in rat skeletal muscle (Breuer and Florini, 1965; Florini and Breuer, 1966). These studies show that direct effects occur by steroids ligand binding to the specific intracellular, steroid receptors and individual steroids may be ligands for multiple types of steroid receptors.

The other type of cellular response is a rapid signaling response that can occur within a matter of seconds to minutes of cell stimulation (Prossnitz et al., 2008). Rapid signaling responses are often associated with growth factor receptors and transmembrane G-protein coupled receptors (GPCR). Steroid ligands bind to the GPCR on the outer surface of the cell membrane. Ligand-activated GPCRs can bind directly to intracellular proteins, resulting in receptor and cellular regulation (Bockaert et al., 2004). One study demonstrated how GPR30 (a GPCR) was involved in estrogen-mediated activation of ERK1/2 (a mitogen-activated protein kinase) in cells lacking classical nuclear estrogen receptors (Filardo et al., 2000). Another study by Kamanga-Solla et al. (in press) suggested that the GPR-30 receptor may be involved in E₂-stimulated IGF-I expression in muscle. These GPCR are related to cellular responses such as calcium mobilization, kinase activation, and nitric oxide production (Prossnitz et al., 2008). One reason this process may be so rapid is that the ligand binding occurs in extracellular space, as apposed to the classical intracellular binding with genomic responses. These studies show that direct steroid activity is a very complex issue that is capable of mediating growth through various courses of action.

One component of anabolic steroid's ability to indirectly stimulate muscle growth is through its effect on insulin-like growth factor-I (IGF-I). In studies, IGF-I has been shown to be a potent stimulator of protein synthesis in skeletal muscle by its role in satellite cells activity, as well as being able to reduce the rate of protein degradation (Florini et al., 1996; Barton-Davis et

al., 1999). With implantation of TBA and E₂ there was an increase in the levels of IGF-I circulating, and longissimus muscles of steers expressed higher levels of IGF-I mRNA when compared to non-implanted steers after 30 to 40 days (Frey et al., 1995; Johnson et al., 1996, 1998a; Dunn et al., 2003; White et al., 2003). It was determined that IGF-I mRNA levels are greater in biopsy samples from the longissimus muscle as early as 12 days after being implanted with TBA + E₂ in steers, when compared to non-implanted steer (Pampusch et al., 2003).

Another way that anabolic steroids achieve increases in skeletal muscle accretion is through increases in skeletal muscle satellite cells. Steers that received an implant containing the combination of TBA and E₂ were found to have a greater number of proliferating satellite cells isolated from skeletal muscle tissue, 24 and 34 hours post implantation, as well as greater rates of proliferation 31 days post implantation (Johnson et al., 1998a). There was also 18% greater fusion of satellite cells from cultures of muscle tissue from the implanted steers versus the non-implanted group. Furthermore, the implanted group had 38% more nuclei found in the myotubes of the fused cultures compared to the non-implanted group (Johnson et al., 1998a). In another study, in vitro methods were used to analyze the direct effect of TBA and E₂ on proliferation of satellite cell cultures that were obtained from non-implanted steers (Kamanga-Sollo et al., 2004). These results found that exposing the satellite cells to 10 nM estradiol or 10 nM trenbolone resulted in rates of proliferation 1.5 times greater than the rate of the control group. One factor that is important to recognize is that in order to overcome the antagonistic action of IGFBP-3 on IGF-I, the media used in the culture was depleted of IGFBP-3 (Kamanga-Sollo et al., 2004). These studies show that anabolic steroids affect growth through various pathways, many of which lead to dramatic effects on the performance of meat producing animals. Furthermore, the accumulation of DNA from satellite cells into muscle fibers is an important aspect of postnatal skeletal muscle growth, which can be altered by the use of anabolic steroids.

Effect of Anabolic Steroids on Performance

Anabolic steroids enhance muscle growth, improve rate of gain, and increase feed efficiency in cattle (Johnson et al., 1996; Pampusch et al., 2003). Heifers administered estrogen only compounds were found to have increases in daily gains by 12 to 16% (Dinnusson et al., 1950). Steers implanted with the combination of TBA and E₂ were found to have improvements in ADG, feed efficiency, longissimus muscle area, and carcass protein, with no effects on DMI or fat accumulation (Johnson et al., 1996; Pampusch et al., 2003). In a study comparing TBA

only, E₂ only, and TBA/E₂ combined, in yearling steers, average daily gain and skeletal muscle protein content were greatest in the group receiving the combination of TBA/E₂, followed by E₂ only, and the weakest response was in TBA only group (Hayden et al., 1992). The combination of TBA/E₂ has also been found to increase rib protein percent and decrease rib fat percent in implanted steers (Lee et al., 1990). Another study by Platter et al. (2003) analyzed the impact of lifetime implant protocols on performance and quality of beef steers. They found that with more repetitive and aggressiveness of implant protocols, there were increases in live and carcass performance; however marbling, tenderness, and consumer acceptability were decreased (Platter et al., 2003). The impact that steroids have on skeletal muscle accretion is well established and accepted, however the methods and mechanisms that elicit the response in vivo is still not fully understood.

β -Adrenergic Agonists

Compounds known as β -AA are capable of eliciting a biological response when bound to a β -AR (Mersmann, 1998). These β -AA belong to the class of compounds known as phenethanolamines (Barnes, 1995), and are either endogenous structures that are naturally synthesized within the body, or exogenous compounds that are synthetically produced outside the body. Two endogenous phenethanolamines are the catecholamines, epinephrine and norepinephrine (Smith, 1998). Both of these compounds vary in their affinity to the β -AR as well as the response they elicit through the receptors. Exogenous compounds are orally administered to elicit a biological response in livestock species. There are two types of β -AA that are utilized to improve growth efficiency and productivity in finishing beef cattle and in swine. Ractopamine-HCl (RH) was approved by the FDA for use in pigs in 1999 as well as in cattle in 2003. The β_1 -AA RH, marketed as PayleanTM, can be orally administered to pigs in the feed up to 10 mg/kg of diet during the last 20.4 to 40.8 kg of weight gain. RH is also marketed as OptaflexxTM, and it can be fed up to 400 mg per head daily to feedlot cattle for the last 28 to 42 days prior to harvest with no withdrawal period. The other type of β -AA that was approved in the United States in 2006 for use in cattle was the β_2 -AA Zilpaterol-HCl (ZH), marketed as ZilmaxTM. It can be fed from 60 to 90 mg per head daily to for the last 20 to 40 days prior to harvest, with a three day withdrawal period. Zilmax is only approved for use in feedlot cattle. There are several other β -AA such as cimaterol, clenbuterol, isoproterenol, L-644,969, and

salbutamol, that are not currently approved for use with finishing cattle in the United States (Moody et al., 2000; Mersmann et al., 1998; Smith, 1998). Each of these β -AA has varying physiological responses in growth rate and lean tissue accretion due to differences in conformational structure.

β -Adrenergic Agonist Receptors

The primary way for β -AAs to directly produce a biological response is to bind to β -adrenergic receptors (β -AR) on the cell surface. Almost all cell types within the mammalian body have β -AR integrated into the plasma membrane (Mersmann, 1998). There are three types of β -AR that have been discovered, and they are referred to as β_1 -, β_2 -, and β_3 -AR. There is approximately 40 to 50% homology between receptor subtypes within a species (Mersmann, 1998). When compared across species there is 75% or greater homology for any of the β -AR subtype (Pietri-Rouxel and Strosberg, 1995). Therefore the number and type of receptors can vary within and between species. The primary receptor found in bovine adipose tissue is the β_2 receptor (Van Liefde et al., 1994). Other studies have determined that the β_2 -AR is the most abundant subtype in both the longissimus dorsi muscle as well as in peri-renal adipose tissue of cattle (Sillence and Matthews, 1994). Furthermore, recent studies on semimembranosus muscle of steers and heifers have shown that the most abundant β -AR mRNA detected was for the β_2 -AR subtype (Winterholler et al., 2006; Sissom et al., 2007a). The β_2 -AA ZH can bind to both the β_1 -AR and β_2 -AR, although it has a greater affinity for the β_2 -AR (Verhoeckx et al., 2005). Across all the types of β -ARs, there exist similar physical characteristics.

The β -AR is comprised of >400 amino acids in a continuous chain that are anchored to the plasma membrane by seven relatively hydrophobic transmembrane domains (Mersmann, 1998). Unlike anabolic steroids, β AA can not pass through the cell membrane, and therefore ligand binding occurs on the extracellular portion of the cell, which activates the intracellular portion of the β -AR/ G_s or β -AR/ G_i complex. The activation of the G_s complex leads to activation of adenylyl cyclase to produce cyclic AMP from ATP. Increases in cAMP activate protein kinase A, which leads to the phosphorylation of the serine/threonine residues of key protein enzymes. This can either cause activation or inactivation of these protein enzymes. One such protein that can be activated is hormone sensitive lipase that is the rate limiting step in lipolysis (Mersmann, 1998). A protein that can be inactivated is acetyl CoA carboxylase which is the rate limiting step in lipogenesis (Liggett and Raymond, 1993). Gene activation can also be

affected by protein kinase A (PKA) phosphorylating transcription factors like cAMP response element binding protein (CREB), which binds to DNA (Mersmann, 1998). Other studies have reported on β -AA effect of increasing in the amount of RNA transcript for the muscle proteins myosin light chain (Smith et al. 1989), α -actin (Helferich et al. 1990; Grant et al. 1993), and calpastatin (Bardsley et al. 1992). These biological responses from the activation of β -AR indicate how tissue deposition and accumulation can be directly affected.

The administration of β -AA appears to increase efficiency of growth by preferentially stimulating skeletal muscle growth compared to adipose tissue. It is thought that β -AA achieve this through decreasing the natural in vivo degradation of protein, and increasing protein translated from RNA. Data suggests that β -AA may contribute to lean muscle mass by acting upon the calpain-calpastatin complex and decreasing protein degradation (Pringle et al., 1993). Steers receiving close arterial infusion of cimaterol had increased calpastatin mRNA (Sun et al., 1994). Myoblast from rats were exposed to cimaterol and there was a decrease in protein degradation, but no effect on protein synthesis (Forsberg and Merrill, 1986). Pigs that were administered ractopamine had an increase in fractional protein synthesis rates and a decrease in fractional protein breakdown rate (Bergen et al., 1989). In a study with steers that were administered clenbuterol, mRNA transcripts of specific muscle proteins increased by 90% in the longissimus muscle (Smith et al., 1995). Another study administered L-644,969 to steers and noticed a 27% reduction in the fractional degradation rate of protein (Wheeler and Koohmaraie, 1992). Ractopamine was found to decrease adipose tissue sensitivity to insulin in rats (Hausman et al., 1989) and in pigs (Liu and Mills, 1990). It has also been observed that chronic treatment of β -AA increases sensitivity to insulin in the soleus muscle of rats (Budohoski et al., 1987). These studies show how β -AA may alter glucose uptake by adipose tissue, and repartition those nutrients to other tissues like muscle, as well as reducing rate of protein turnover, and ultimately increasing total protein accumulation.

It has also been hypothesized that β -AA may have an indirect effect on hypertrophy through increased blood flow. Steers that were administered cimaterol through close arterial infusion increased the rate of blood flow and extraction of essential amino acids from the circulation in the hind limb where it was administered (Byrem et al., 1996). Similar results were reported in cattle administered clenbuterol (Eisemann and Huntington, 1993) and in lambs administered clenbuterol (Aurousseau et al., 1993). These studies show that β -AA do have

indirect tissue altering ability by increasing blood flow which could result in more nutrients and factors being made available to specific tissues. Another possible indirect effect that has been studied is β -AA effect on satellite cell activity.

There has been little evidence to show that β -AA have any effect on satellite cell activity. In a study conducted on rats, DNA synthesis was inhibited in the soleus muscle, but there were no significant alterations in the extent of muscle hypertrophy or myofibril content (Fleckman et al., 1978). When lambs were administered cimaterol, the semitendinosus muscle had greater levels of protein and RNA content, and a decrease in DNA concentrations after 7 weeks of administration, which then returned to normal after 12 weeks of administration (Beermann et al., 1987). In a similar study, Kim et al. (1987) had the same conclusion. When O'Connor et al. (1991) conducted a study with sheep administered cimaterol for 3 weeks they noticed a 25% increase in RNA concentrations, 85% increase in total mass of RNA, and a 30% increase in total weight gain. At the same time there was a 42% reduction in DNA concentration, and the total DNA content was unchanged (O'Connor et al., 1991). Sissom et al. (2007b) cultured bovine satellite cells in vitro in media containing ZH, and no changes were detected in satellite cell proliferation. In an in vivo study with cull beef cows administered RH, there were no changes in satellite cell numbers or fiber-associated nuclei (Gonzalez et al., 2007). These studies suggest that stimulation of satellite cell activity was not an essential aspect of this muscle hypertrophy. Furthermore, β -AA did not affect satellite cell proliferation or incorporation into the growing muscle fibers. Therefore it is possible that β -AA can stimulate muscle hypertrophy, and elicit a response without stimulating satellite cell activity. This phenomenon brings to light the possible additive effects of β -AA administered in combination with anabolic steroids, which, as discussed earlier, have been found to stimulate satellite cells. An important factor contributing to possible additive effects is the ability of β -AA to affect protein synthesis and degradation. An indicator of this biological activity can be measured through the gene expression of the muscle specific protein being synthesized. As discussed earlier, one of the major proteins involved in protein synthesis is myosin, and more specifically MHC.

Feeding cimaterol to lambs reduces type I MHC fibers, and muscle hypertrophy is primarily attributed to type II fibers (Beerman et al., 1987). Clenbuterol was administered to rats and type II fibers were found to increase, whereas type I fibers decreased in the soleus muscle (Zeman et al., 1988). In a similar study with rats administered clenbuterol, there was a decrease

in the type I MHC and a high amount of type IIX MHC present, which was not detected in the control group. There was also a decrease in the types I, IIA, and IIX in the diaphragm muscle, but an increase in type IIB (Polla, et al., 2001). Another study evaluated the effects of ractopamine in pigs and found that types IIA and IIX decreased, however type IIB was found to increase (Depreux et al., 2002; Gunawan et al., 2007). These studies imply that β -AA feeding stimulates fibers to transition from slow to faster phenotypes, and faster fiber types of MHC are larger in diameter than slower fiber types (Taylor, 2004). This could explain the increase in total muscle size that has been observed with administration of β -AA.

Effect of β -Adrenergic Agonist on Performance

Different species of animals appear to have different responsiveness to β -AA (Anderson et al., 1991). Ruminants, sheep and cattle, have a greater response to β -AA compared to swine, and poultry appear to be the least responsive livestock species (Moody et al., 2000). The low responsiveness in chickens has been attributed to the high genetic selection for growth potential in broilers, which show they may be approaching their biological maximum growth rate (Mersmann, 1998). This could explain greater responses observed in cattle and sheep that have not been so rigorously selected for growth potential.

Feeding of β -AA enhances carcass leanness, improves growth rates, and decreases feed consumption in cattle and sheep (Plascencia et al., 1999; Salinas-Chavira et al., 2004). Most studies evaluating β -AA have also implanted cattle with an anabolic steroid. This is to reflect the regimen of growth promoters that are typically used in the conventional beef finishing industry. Steers implanted with Revalor-S and administered RH were found to have increased ADG, G:F, HCW, and LM area, with no changes in feed intake, dressing percentage, USDA yield grade, or quality grade (Winterholler et al., 2007). A similar study that evaluated the performance of heifers implanted with Finaplix-H and administered RH, found an increase in ADG, G:F, HCW, and LM; improved yield grade; and decreased 12th rib fat depth (Sissom et al., 2007a). The β_2 -AA ZH is expected to have similar, if not greater, effect on performance relative to RH due to type of β -AA and affinity to the β_2 -AR which is the most abundant in skeletal muscle. Steers implanted with Revalor-S and administered ZH were found to have an increase in ADG, G:F, HCW, dressing percentage, and LM area. There was also a decrease in feed intake, 12th rib fat depth, marbling, KPH and yield grade (Vasconcelos et al., 2008). Another study showed that steers implanted with Synovex Plus and administered ZH had greater ADG, HCW, G:F, and

carcass yield, but no effect on feed intake (Avendaño-Reyes et al., 2006). In another study, steers that were implanted with Revalor and administered ZH were found to have similar performance, but with no differences in feed intake, 12th rib fat depth, or marbling (Plascentia et al., 1999). It is important to note that each of these studies used different breeds as well as variations in physiological endpoints that these animals were harvested at. Because almost all studies evaluating ZH in finishing cattle have preceded ZH administration with a steroid implant, it is difficult to accurately determine what the performance response to the β_2 -AA would be in non-implanted cattle. Therefore further studies are needed to determine the extent of performance that can be attributed to ZH.

Anabolic Steroid Use Combined with a β -Adrenergic Agonist

Little research has looked into the combined effects of anabolic steroids and β -AA on the performance and carcass characteristic in cattle. Studies have compared the effects of steroid hormones and bovine growth hormone on performance and carcass characteristics in cattle. These studies reported that steroid hormones and growth hormone have additive improvements on performance in cattle (Enright et al., 1990; Preston et al., 1995). Differences in the mechanisms of action stimulating skeletal muscle accretion with β -AA and steroid hormones have made us question the extent of additive effects on growth, from combined treatment. Animal performance is improved by the direct effects of anabolic steroids binding with intracellular receptors and eliciting a response through regulation of transcription for tissue synthesis and degradation. They also have an indirect effect by stimulating satellite cell proliferation which increases the overall capacity for hypertrophic growth. Administration of β -AA directly affects the extent that DNA transcribes RNA and in turn translates protein. Due to this, it has been hypothesized that anabolic steroids elicit biological responses that synthesize the “machinery” that is necessary for hypertrophy, and β -AA improve the efficiency with which that “machinery” is capable of accumulation of protein through hypertrophy. Therefore, the increased growth response could be synergistic, if not additive, in nature with the combination of these two growth promoters in finishing beef cattle.

Conclusion

As the world population continues to grow, the demand on agriculture to produce sufficient food is becoming more pronounced. Our dependence on photosynthesis to provide the world with food, fabric, and fuel has driven science to evolve the most efficient practices to utilize every aspect of this process. One practice the beef cattle industry in the United States has adopted is the use of exogenous growth promoting agents to increase the efficiency that nutrients are converted to edible protein. Examples of this are seen in the improved performance of finishing beef cattle that are administered anabolic steroids and β -adrenergic agonist. The biological processes that enable these changes in animal growth are complex and intriguing; however, science has worked to provide a better understanding by investing in research to focus on this issue. With demands for red meat products increasing and the natural resources available for its production decreasing, it is important that we consider, and utilize practices that will efficiently and safely provide the world with wholesome red meat beef products.

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CHAPTER 2 - Zilpaterol-HCl alters myosin heavy chain mRNA abundance in finishing steers.¹

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Abstract

This experiment investigated the effects of zilpaterol-HCl (ZH) and the steroidal implant Revalor-S (RS; 120 mg trenbolone acetate and 24 mg estradiol-17 β) on finishing steer performance and the mRNA concentration of β -adrenergic receptors (β -AR) types I and II, calpastatin, and types I, IIA, and IIX myosin heavy chain (MHC) isoforms. A total of 2,279 feedlot steers weighing 426 ± 6.4 kg were administered no implant or RS on d 0, and fed either 0 or 8.3 mg ZH/kg of diet DM during the last 30 d with a 3 d withdrawal. Treatments were randomly assigned to 24 pens ($n = 6$ pens/treatment). At harvest, semimembranosus muscle tissue was excised for RNA isolation from 4 carcasses per pen. No interactions were detected for any of the variables measured in the experiment. Administration of ZH during the last 30 d of the feeding period increased ($P < 0.01$) ADG, G:F, HCW, and LM area; decreased ($P < 0.01$) 12th rib fat depth and marbling; and improved ($P < 0.01$) yield grade. Treatment had no effect on $\beta 1$ -AR mRNA levels, but there was an increase ($P = 0.01$) in $\beta 2$ -AR mRNA levels due to ZH inclusion. Myosin heavy chain-I (MHC-I) mRNA levels were unaffected by treatment. For MHC-IIA mRNA concentrations, administration of RS tended ($P = 0.08$) to increase mRNA levels, whereas ZH feeding the last 30 d tended ($P = 0.08$) to decrease mRNA levels for this isoform of myosin. Feeding ZH the last 30 d prior to harvest, increased ($P < 0.01$) mRNA concentrations of MHC-IIX in semimembranosus muscle of steers. These data indicate the combined use of ZH and RS additively contributes to live and carcass gain in finishing feedlot steers. In addition, ZH feeding changes the mRNA levels of MHC isoforms to a faster, more glycolytic fiber type in bovine skeletal muscle. These changes in mRNA concentrations of MHC isoforms due to ZH feeding could be affecting skeletal muscle hypertrophy.

Introduction

Zilpaterol-HCl (ZH) is an orally active β -adrenergic agonist (β -AA) approved for use in finishing beef cattle in the United States. Inclusion of ZH in cattle feed the last 20 to 40 d, improves ADG, feed efficiency, carcass yield grade, HCW, LM area and dressing percentage in finishing steers (Avendaño-Reyes et al., 2006; Vasconcelos et al., 2008). These biological effects are a result of ZH binding to a β -adrenergic receptor (β -AR) located on the cell surface of tissues including skeletal muscle and adipose tissue (Mersmann, 1998). There are 3 sub-types of β -AR (β 1, β 2, and β 3) on most mammalian cells with β 2-AR being the most abundant receptor sub-type in bovine skeletal muscle and adipose tissue (Sillence and Matthews, 1994). Zilpaterol-HCl can bind to both the β 1-AR and β 2-AR, with a greater affinity for β 2-AR (Verhoeckx et al., 2005). The β -AA increase skeletal muscle hypertrophy. These improvements in skeletal muscle hypertrophy are a result of changes in protein synthesis and degradation rates, whereas in adipose tissue they promote lipolysis (Beermann, 2002; Birkelo, 2003; Verhoeckx et al., 2005).

Anabolic steroidal implants containing the combination of trenbolone acetate (TBA) and estradiol-17 β (E2) have been reported to improve feedlot performance and stimulate carcass protein accretion in feedlot steers (Johnson et al., 1996; Pampusch et al., 2003). Data also indicates that TBA + E2 implantation increases proliferation, and fusion of muscle satellite cells, which may be an important mechanism by which anabolic steroids enhance muscle hypertrophy (Johnson et al., 1998; Johnson and Chung, 2007).

Currently, there are no data on the comparative efficacy of these 2 distinct types of growth promotants in the feedlot steers in the United States. The purpose of this study was to investigate the effects of ZH administration in combination with a steroidal implant containing TBA and E2 on steer performance and the mRNA abundance for β 1-AR; β 2-AR; calpastatin; and myosin heavy chain (MHC) types -I, -IIA, and -IIX.

Material and Methods

The following experiments were a collaboration of Intervet Inc. (Millsboro, DE), Texas Tech University, and Kansas State University. Experimental procedures with cattle were in compliance with the guidelines stated in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS 1999).

Animals

English X Continental yearling steers ($n = 2,279$), with initial BW of 426 ± 6.4 kg, were utilized in this study. All animals were housed at a commercial research facility in Texas in soil-surface pens. At approximately d -61, all animals were administered a Component TE-IS (16 mg estradiol and 80 mg TBA; Vetlife Inc., West Des Moines, IA) implant. Prior to allotment, animals were weighed and ultrasound was used to estimate empty body fat (EBF), so that pens could be stratified by EBF. Extreme animals in terms of BW or EBF were removed from the trial. Furthermore, animals with missing EID tags or with visual performance or health problems were also eliminated from the trial.

Experimental Design, Treatment, and Pen Assignment

Four treatments were arranged in a 2 x 2 factorial design with the main effects of feeding ZH (0 or 8.38 mg/kg; Zilmax; Intervet, Inc., Millsboro, DE) for the last 30 d on feed with a 3 d withdrawal (in accordance with FDA regulations), and a terminal implant of Revalor-S (RS; 120 mg TBA and 24 mg E2; Intervet, Inc., Millsboro, DE) 91 d before harvest or no implant. Treatments were randomly assigned to each pen ($n = 24$) with approximately 100 steers per pen, as follows: 1) no RS or ZH (CON) or 2) only ZH [ZH], 3) only RS (RS) or 4) RS and ZH [ZH+RS]. The different treatment groups were designed to be reflective of the typical commercial finishing period, and to evaluate the impact of inclusion of ZH in the diet. The ZH was included by means of a ground corn-based premix (Table 2.1). When the study was conducted ZH was not approved by the FDA to be fed in combination with monensin (Rumensin; Elanco Animal Health, Indianapolis, IN) or tylosin (Tylan; Elanco Animal Health, Indianapolis, IN); therefore, during the ZH feeding period, these feed additives were removed from the diets of the treatments receiving ZH for 30 d, then administered again during the 3 d withdrawal to all treatments. The diet ingredient and chemical compositions are provided in Table 2.1. The diet was formulated to meet or exceed NRC (1996) requirements for nutrients. Throughout this study a clean bunk method was implemented so that all feed was consumed each day without limiting feed available. Feed allocation (increase or decrease in daily amount) was based on the time bunks were empty and visual appraisal of cattle appetite aggression. Cattle were fed 3 times daily with approximately equal distribution of the day's feed across the 3 feedings. Pens were fed in the same order each day. At the end of the feeding period BW was

measured, and the industry standard, 4% pencil shrink, was used to calculate final BW. All animals were transported approximately 193 km to a commercial abattoir to be harvested.

Sample Preparation and RNA Isolation

Within 10 min of slaughter at the abattoir, a muscle sample was collected from the semimembranosus muscle of 4 randomly selected steers per pen. The samples were rapidly frozen in liquid N₂ and shipped to Kansas State University for analysis. Total RNA was isolated from muscle samples with Tri Reagent (Sigma, St. Louis, MO). Briefly, the semimembranosus muscle tissue (200 mg) was transferred to a steel mortar bowl cooled by liquid N₂. The samples were homogenized by using a sterile pestal in liquid N₂, and Tri Reagent (2 mL) was added to the ground tissue sample. Muscle tissue (1 mL) in Tri Reagent was incubated at room temperature for 5 min. After incubation, chloroform (Sigma, St. Louis, MO) was added, and the samples were centrifuged for 15 min at 12,000 x g at room temperature. After centrifugation, the upper aqueous phase was removed and transferred to a new microcentrifuge tube. Isopropanol (Sigma, St. Louis, MO) was added, and incubated at room temperature for 5 min. Then the samples were centrifuged for 10 min at 12,000 x g to isolate the RNA pellet. The isopropanol was then removed and the RNA pellet was suspended in 70% ethyl alcohol (1 mL) and stored at -80°C.

The RNA pellet was treated to remove any contaminating genomic DNA by using the DNA-free kit (Ambion, Austin, TX). The RNA concentration was determined by absorbance at 260nm. The integrity of the RNA was determined by gel electrophoresis. Total RNA with ethidium bromide was loaded onto a 1% agarose gel to separate and visualize the 28S and 18S ribosomal RNA (18S). Total RNA (1 µg) was then reverse-transcribed to produce the first-strand complementary DNA (cDNA) using TaqMan Reverse Transcription Reagents and Multi-Scribe Reverse Transcriptase (Applied Biosystems, Foster City, CA) following the protocol recommended by the manufacturer. Random hexamers were used as primers in cDNA synthesis.

Real-Time Quantitative PCR

Real-time quantitative PCR was used to measure β 1-AR, β 2-AR, calpastatin, MHC-I, MHC-IIA, and MHC-IIB quantitative gene expression relative to the quantity of ribosomal protein S9 (RPS9) in total RNA isolated from muscle tissue. Measurements of the relative quantity of cDNA was performed by using TaqMan Universal PCR Master Mix (Applied

Biosystems, Foster City, CA), 900 nM of the appropriate forward and reverse primers, 200 nM of the appropriate TaqMan detection probe, and 1 μ L of the cDNA mixture. The bovine-specific β 1-AR, β 2-AR, calpastatin, MHC-I, MHC-IIA, and MHC-IIB forward and reverse primers and TaqMan detection probes (Table 2.2) were synthesized by using published GenBank sequences. Custom RPS9 (Genbank Accession No. DT860044) rRNA primers and probes were used as an endogenous control. The ABI Prism 7000 detection system (Applied Biosystems, Foster City, CA) was used to perform the assay by using the recommended thermal cycling variables from the manufacturer (50 cycles of 15 s at 95°C and 1 min at 60°C). The RPS9 rRNA endogenous control was used to normalize the expression of β 1-AR, β 2-AR, calpastatin, MHC-I, MHC-IIA, and MHC-IIB. The data were expressed as relative units.

Statistical Analysis

Data were analyzed as a 2 x 2 factorial in a randomized complete block design with PROC MIXED (SAS Inst. Inc., Cary, NC). Pen served as experimental unit for all feedlot, carcass characteristics, and gene expression data. Treatment and interaction means were analyzed and separated ($P < 0.05$) with the least significant difference procedure of SAS and Fisher's exact test.

Results and Discussion

Effect of ZH and RS on Performance and Carcass Characteristics

Performance data represent the entire 91-d period from RS implant until harvest. All performance and carcass data are shown in Table 2.3. The RS treatment increased ($P < 0.01$) ADG and G:F, and increased ($P = 0.02$) DMI by 2.2%. These results are similar to previous studies on steroidal implants showing improvements on finishing steer performance and feed efficiency (Bartle et al., 1992; Johnson et al., 1996; Guiroy et al., 2002; Pampusch et al., 2003). The increase in DMI was in agreement with previous studies utilizing estrogen implants that reported an increase in DMI (Rumsey et al., 1992).

Carcass yield improved with increases ($P < 0.01$) in HCW and LM area as well as an increase ($P < 0.05$) in dressing percentage as a result of RS treatment (Table 2.3). There was a change in quality grade, with marbling scores decreasing ($P < 0.01$) with RS treatment; however, no differences were observed for 12th rib fat depth. Our results are similar to a previous study

that found improvements in performance with implant use; however quality grade also decreased with increased implant use in steers (Platter et al., 2003). These results show that RS implant improves finishing steer performance through increased efficiency of nutrient conversion to skeletal protein; however it does have a negative impact on carcass quality grade due to decreases in final adipose amounts.

The ZH treatment increased ($P < 0.01$) ADG, G:F, HCW, dressing percentage, and LM area; and decreased ($P < 0.01$) 12th rib fat depth and marbling scores. With the ZH treatment, there was no effect on DMI. These results show that ZH improves performance, and enhances carcass protein accumulation without stimulating DMI, leading to improved efficiency with which dietary nutrients are converted to gain. However, there is a reduction in adipose tissue. These results are similar to other studies that have detected increases in performance and carcass lean tissue accumulation in cattle and sheep (Plascencia et al., 1999; Salinas-Chavira et al., 2004).

It is known that the response of ZH in cattle is mediated through the β -AA binding and activation of β -AR. Zilpaterol hydrochloride functions mainly through the β_2 -AR which predominates in skeletal muscle and adipocytes in cattle (Birkelo, 2003). Once the receptors are activated, intracellular actions are mediated through cyclic AMP and subsequent activation or deactivation of key enzymes. The net effect is to direct nutrients in support of enhanced protein synthesis (Birkelo, 2003). It has been demonstrated that β -AA promote triacylglycerol degradation in the adipocyte and block fatty acid synthesis in vitro (Mersmann, 1998). Research conducted in vivo with heifers showed that clenbuterol administration reduced adipocyte cell volume, suggesting the depression in lipid deposition was due to the inhibition of pivotal enzymes necessary for de novo fatty acid biosynthesis (Smith et al., 1987). Our understanding of the primary method with which ZH elicits a biological response supports the results observed in the current study, as well as the concept that ZH acts as a repartitioning agent to disrupt the ability of adipose tissue to utilize nutrients, making more nutrients available for muscle hypertrophy.

The cattle receiving the combination ZH+RS treatment had the greatest increase in ADG and G:F that appear to be additive when compared to the individual treatments with ZH or RS. The ZH+RS treatment also had an additive increase in HCW, LM and dressing percentage when compared to individual treatments of ZH or RS. Marbling scores, 12th rib fat depth, and KPH

were additively decreased for the ZH+RS treatment when compared to individual treatments of ZH or RS. There is little published research available that compares the effects of implants and ZH in cattle, however, these results are supported by similar studies that found increases in ADG, G:F, HCW, dressing percentage, and LM area; decreases yield grade, 12th rib fat depth, and marbling scores in finishing steers that were administered RS (120 mg TBA and 24 mg E2) and received feed inclusion of ZH (8.33 mg/kg) for 0, 20, 30, or 40 d (Vasconcelos et al., 2008). Similarly, Avendaño-Reyes et al. (2006) reported increased ADG, G:F, HCW, and LM area; decreased 12th-rib fat depth and no difference in DMI by steers that received Synovex Plus (200 mg TBA and 28 mg estradiol benzoate) 60 d before ZH administration (Avendaño-Reyes et al., 2006).

These results support our findings that steroid implant and inclusion of ZH in typical finishing steer rations, improve feed efficiency and animal performance without affecting DMI. Furthermore, the largest improvements in performance and efficiency are achieved, and appear to be completely additive, when steers are administered a steroidal implant prior to inclusion of ZH in the ration; however carcass quality decreases to a similar extent. The additive nature of the data implies that these 2 growth promotants may be working through distinct mechanisms to enhance lean tissue deposition.

Real Time PCR

In performing the real time PCR, we initially determined quantitative values of each gene of interest relative to the quantity of the housekeeping gene (HKG) 18S rRNA to normalize the data. From our analysis, we determined there was a treatment effect ($P = 0.05$) of ZH on 18S rRNA concentrations. Total RNA isolated from muscle samples of ZH cattle had greater levels of 18S rRNA. Therefore, we used RPS9 as our HKG. In a previous study, various HKG were tested for variation with bovine hepatic tissue from animals of varying physiological and dietary experimental conditions. It was determined that of those tested the HKG RPS9 was the most stable relative to the various experimental types of hepatic tissue (Janovick-Guretzky et al., 2007). Following analysis of our study it was determined that no effect ($P = 0.43$) was detected with the HKG RPS9 for any of the treatment groups. From this discovery, the question arises about ZH possibly having an effect on ribosomal RNA gene expression. This would be another indicator that protein synthesis may be altered with ZH administration.

Effect of ZH and RS on Semimembranosus Muscle β 1-AR, β 2-AR, and Calpastatin mRNA Concentrations

The mRNA concentrations of β -AR and calpastatin are shown in Figures 2.1 through 2.3. There was no effect from any treatments on the expression of β 1-AR mRNA. The ZH treatment increased ($P = 0.01$) mRNA expression of the β 2-AR, however there was no other treatment effects on the β 2-AR. No significant treatment effects were observed for calpastatin mRNA concentrations. These findings are similar to a study by Sissom et al. (2007) in heifers initially implanted with 80 mg TBA and 8 mg E2, and re-implanted with 200 mg TBA 96 d prior to feeding the β -AA, ractopamine-HCl (RH), for 28 d. These authors reported no change in β 1-AR mRNA levels, and a tendency to increase β 2-AR mRNA levels, due to β -AA administration (Sissom et al., 2007). In a similar study by Winterholler et al. (2007), with steers implanted with RS and administered RH, there was no β -AA treatment effect on the expression of β 1-AR mRNA levels. However there was a tendency to increase β 2-AR mRNA levels (Winterholler et al., 2007). They further determined in vitro that primary bovine muscle cultures, in response to RH, showed an increase ($P < 0.001$) in the β 2-AR mRNA level during differentiation, but no effect on β 1-AR mRNA levels (Winterholler et al., 2007). These studies support our current findings that administration of a β -AA in steers can alter expression of skeletal muscle β 2-AR mRNA. The differences in the amount of change between these studies could be due to the differences in the type and duration of β -AA administered. Additionally, our study determined the expression of β 2-AR was nearly 1000 times greater than the expression of β 1-AR, which suggests that the β 2-AR is the most abundant β -AR sub-type in the semimembranosus muscle of steers. This corresponds with previous research that determined the β 2-AR mRNA is the most abundant mRNA sub-type in semimembranosus of heifers and steers (Sissom et al., 2007; Winterholler et al., 2007) as well as β 2-AR being the most abundant receptor in bovine LM and peri-renal adipose tissue (Sillence and Matthews, 1994).

Effect of ZH and RS on Semimembranosus Muscle Type-I, -IIA, and -IIX MHC mRNA Concentrations

Administration of ZH and RS increases lean tissue deposition. From these findings we hypothesized that the expression of myosin isoforms had the potential to be altered as a result of the changes in lean tissue deposition, due to ZH and RS administration. The gene expression results for MHC are shown in Figures 2.4 through 2.6. There was no effect on the expression of

MHC-I mRNA for all treatments. The ZH treatment had a tendency to decrease ($P = 0.08$) MHC-IIA mRNA, and the RS treatment had a tendency to increase ($P = 0.08$) the expression of MHC-IIA mRNA. There was an increase ($P < 0.01$) in the expression of MHC-IIX mRNA as a result of the ZH treatment, but the RS treatment had no effect. Previous studies have shown a decrease in abundance of slower fiber types IIA and IIX MHC protein, and an increase in abundance of the fiber type IIB MHC in porcine longissimus and semitendinosus muscles, due to β -AA administration, (Dupreux et al., 2002). Within the bovine specie one study attempted to detect the fiber type IIB MHC mRNA, which is the fastest, most glycolytic fiber type, but none was detected in bovine skeletal muscle (Chikuni et al., 2004). This implied that the type IIX MHC mRNA is the fastest, most glycolytic myosin fiber type of mRNA expressed in cattle. For this reason we did not run an analysis on the expression of type IIB MHC mRNA. In another study rats were administered clenbuterol, and soleus muscle fiber types of MHC showed a transition from a slower, anaerobic to faster, aerobic fiber types (Polla et al., 2001; Zeman et al., 1988). One study showed the transition in muscle fiber types as it relates to increasing physiological maturity in cattle. Solomon et al. (1986) used bulls to show that increasing physiological maturity increased slow-twitch-oxidative (SO) fiber followed by a plateau, decreased fast-twitch-oxidative-glycolytic (FOG) fibers, and increased fast-twitch-glycolytic (FG) fibers. Another study by Lefaucheur et al. (2004) analyzed difference in myosin isoforms and muscle fiber types in pig breeds that differ dramatically in muscle growth by hypertrophy. This study found Meishan pigs, which have lower hypertrophy characteristics, had a greater abundance of MHC-I isoforms and SO fibers and a lower abundance of MHC type -IIA, -IIX, and -IIB isoforms and fewer FOG and FG fiber types when compared to the Large White pigs, which exhibit high muscle hypertrophy characteristics (Lefaucheur et al., 2004). In another study cull cows were utilized to measure the effects of TBA and RH on type -I and -II LM fibers (Gonzalez et al., 2007). They found that TBA and RH increased the cross sectional area (CSA) and fiber diameter of type I fibers with the greatest increase measured in the TBA + RH treatment; however there was no effect on the type II fibers. The lack of effect on the type II fibers could be attributed to not separating the different isoforms of the type II fibers, as well as the effect of the level of physiological maturity on the capacity for muscle hypertrophy (Gonzalez et al., 2007). Another study was conducted to analyze the effects of RH on skeletal muscle gene expression in pigs (Gunawan et al., 2007). They found that RH differentially induced expression of the type IIB

MHC gene (the fastest, most glycolytic isoform in swine skeletal muscle) at the expense of the other isoforms. The altered expression of mRNA for the translation of more glycolytic, fast twitch fiber type IIX MHC due to ZH administration suggests that ZH is capable of altering protein synthesis; furthermore ZH may decrease the amount of mRNA available for translation of the more oxidative, slower fiber type IIA of MHC.

General Conclusion

These results indicate that ZH improved animal performance and increased lean tissue accumulation in finishing steers. When the steroidal implant RS was administered in finishing steers prior to inclusion of ZH in the diet, there were additive improvements on performance and lean carcass characteristics. In addition, there was an increase in the expression of β 2-AR mRNA due to ZH administration. Finally, ZH feeding elicited a differential response in the mRNA abundance of MHC by causing a transition away from slower fiber types and increasing faster fiber types, which could be a consequence of altered protein synthesis and degradation in bovine skeletal muscle.

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Figures and Tables

Figure 2.1. β_1 -Adrenergic receptor (β_1 -AR) mRNA abundance in bovine semimembranosus muscle collected from steers 10 min post-slaughter. Four animals per pen were used in the analysis (6 pens/treatment). Treatments consist of 1) control (CON), 2) zilpaterol hydrochloride (ZH; 8.38 mg/kg), 3) Revalor-S (RS; 120 mg TBA and 24 mg E₂), or 4) ZH and RS (ZH+RS). There was no main effect or interaction of treatments on the expression of β_1 -AR mRNA. Error bars are SEM.

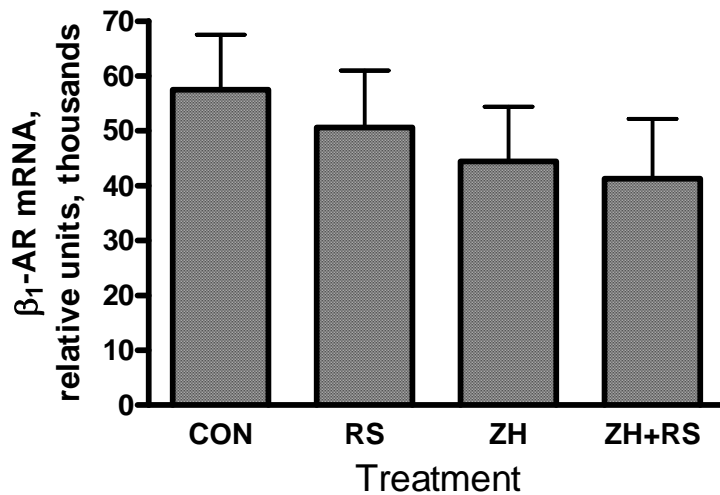


Figure 2.2. β_2 -Adrenergic receptor (β_2 -AR) mRNA abundance in bovine semimembranosus muscle collected from feedlot steers 10 min post-slaughter. Four animals per pen were used in the analysis (6 pens/treatment). Treatments consist of 1) control (CON), 2) zilpaterol hydrochloride (ZH; 8.38 mg/kg), 3) Revalor-S (RS; 120 mg TBA and 24 mg E₂), or 4) ZH and RS (ZH+RS). There was no interaction of treatments on the expression of β_2 -AR mRNA. The main effect of ZH administration increased ($P = 0.01$) the expression of β_2 -AR mRNA, but there was no effect due to RS administration. Error bars are SEM.

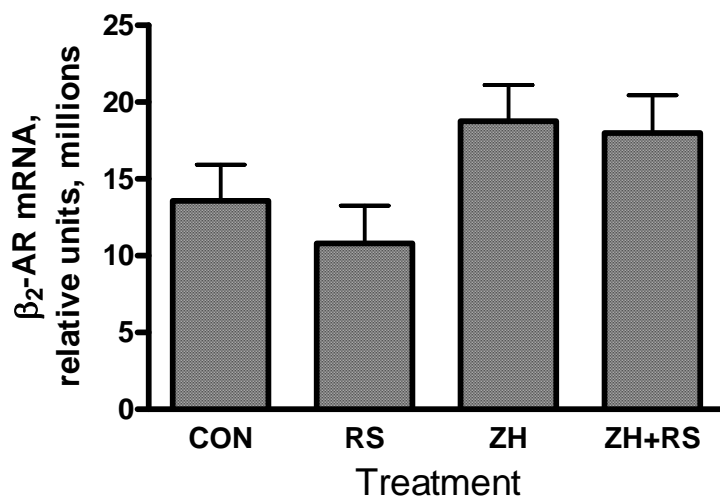


Figure 2.3. Calpastatin mRNA abundance in bovine semimembranosus muscle collected from feedlot steers 10 min post-slaughter. Four animals per pen were used in the analysis (6 pens/treatment). Treatments consist of 1) control (CON), 2) zilpaterol hydrochloride (ZH; 8.38 mg/kg), 3) Revalor-S (RS; 120 mg TBA and 24 mg E₂), or 4) ZH and RS (ZH+RS). There was no main effect or interaction of treatments on the expression of calpastatin mRNA. Error bars are SEM.

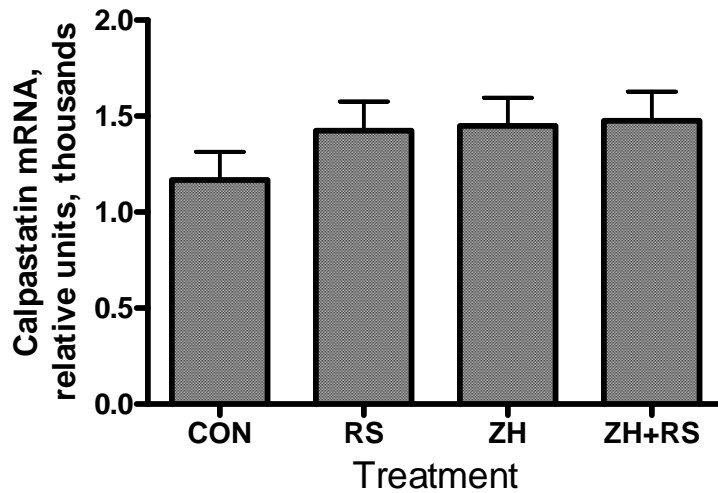


Figure 2.4. Type I myosin heavy chain (MHC-I) mRNA abundance in bovine semimembranosus muscle collected from feedlot steers 10 min post-slaughter. Four animals per pen were used in the analysis (6 pens/treatment). Treatments consist of 1) control (CON), 2) zilpaterol hydrochloride (ZH; 8.38 mg/kg), 3) Revalor-S (RS; 120 mg TBA and 24 mg E₂), or 4) ZH and RS (ZH+RS). There was no main effect or interaction of treatments on the expression of MHC-I mRNA. Error bars are SEM.

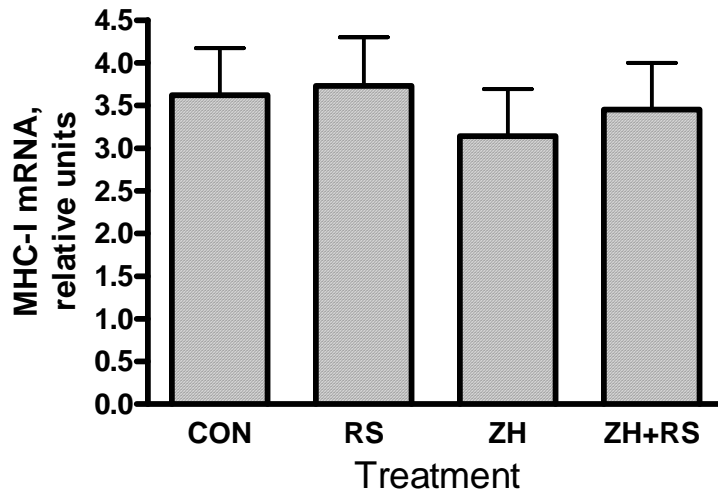


Figure 2.5. Type IIA myosin heavy chain (MHC-IIA) mRNA abundance in bovine semimembranosus muscle collected from feedlot steers 10 min post-slaughter. Four animals per pen were used in the analysis (6 pens/treatment). Treatments consist of 1) control (CON), 2) zilpaterol hydrochloride (ZH; 8.38 mg/kg), 3) Revalor-S (RS; 120 mg TBA and 24 mg E₂), or 4) ZH and RS (ZH+RS). There was no interaction of treatment on the expression of MHC-IIA mRNA. There was a tendency for RS to increase ($P = 0.08$), and a tendency for the ZH to decrease ($P = 0.08$) the expression of MHC-IIA mRNA. Error bars are SEM.

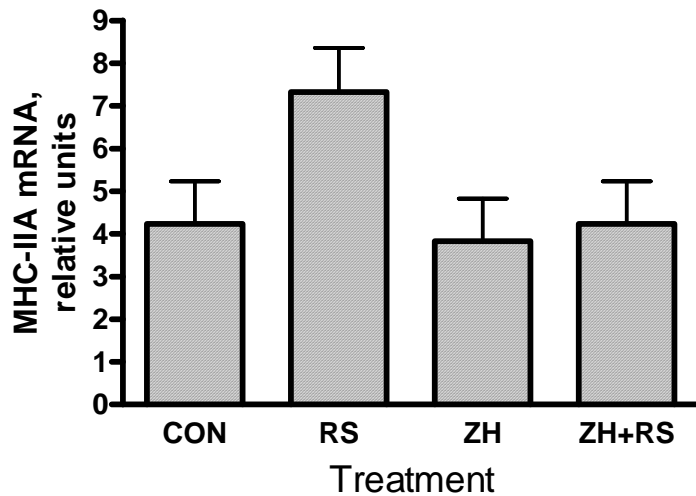


Figure 2.6. Type IIX myosin heavy chain (MHC-IIX) gene expression in bovine semimembranosus muscle collected from feedlot steers 10 min post-slaughter. Four animals per pen were used in the analysis (6 pens/treatment). Treatments consist of 1) control (CON), 2) zilpaterol hydrochloride (ZH; 8.38 mg/kg), 3) Revalor-S (RS; 120 mg TBA and 24 mg E2), or 4) ZH and RS (ZH+RS). There was no interaction of treatment on the expression of MHC-IIX mRNA. Administration of ZH increased ($P < 0.01$) the expression of MHC-IIX mRNA for the ZH and the ZH+RS treatments; however there was no effect of RS administration. Error bars are SEM.

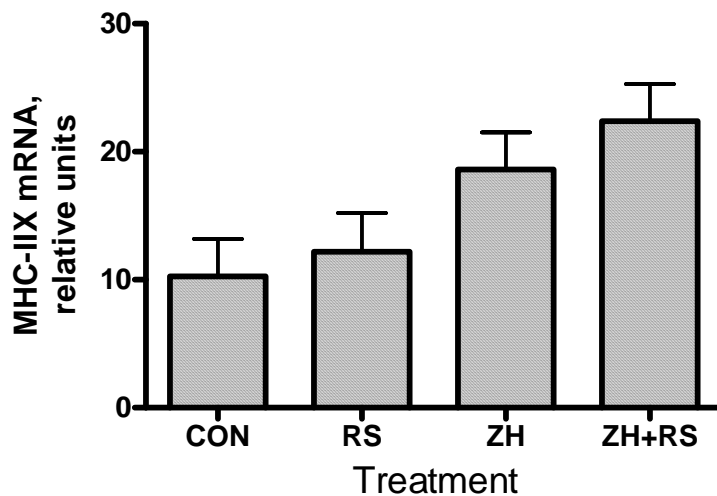


Table 2.1 Composition and analyzed nutrient content (DM basis) of the finishing diet¹

| Item | Treatments ¹ | |
|---------------------------------------|-------------------------|-----------------|
| | Control ² | ZH ³ |
| Flaked corn | 27.55 | 27.55 |
| High moisture corn | 20.00 | 20.00 |
| Wet corn gluten feed | 25.00 | 25.00 |
| Tallow | 2.95 | 2.95 |
| Corn silage | 19.60 | 19.60 |
| Finisher supplement ⁴ | 3.65 | 3.65 |
| Control microingredients ⁵ | 1.25 | - |
| ZH microingredients ⁶ | - | 1.25 |
| Nutrient levels, DM basis | | |
| DM, % | 62.46 | 62.46 |
| CP, % | 12.87 | 12.87 |
| NPN, % | 1.52 | 1.52 |
| ME, Mcal/kg | 3.37 | 3.37 |
| NDF, % | 18.99 | 18.99 |
| Calcium, % | 0.72 | 0.72 |
| Phosphorus, % | 0.46 | 0.46 |

¹Diets represent feed for the last 33 d on feed; from the start of the zilpaterol-HCl (ZH) administration until harvest.

²Treatments not receiving ZH inclusion in the diet.

³Treatment receiving ZH inclusion in the diet.

⁴The finisher supplements contained (DM basis): 58.43% wheat middlings; 7.25% urea; 5.35% salt; 27.20% limestone; 0.50% choice white grease; 0.07% vitamin premix; and 1.20% trace mineral premix.

⁵Added at the time of diet preparation to provide monensin at 30.86 mg/kg of diet DM and tylosin at 9.70 mg/kg of diet DM.

⁶Added at the time of diet preparation to provide zilpaterol hydrochloride at 8.38 mg/kg of diet DM.

Table 2.2 Sequences for bovine-specific PCR primers and Taqman probes for β_1 - and β_2 -adrenergic receptors; calpastatin; types I, IIA, and IIX myosin heavy chain mRNA; and RPS9.

| Item | Sequence (5' to 3') |
|--|--|
| β_1 -adrenergic receptor (accession no. AF188187) | |
| Forward | GTGGGACCGCTGGGAGTAT |
| Reverse | TGACACACAGGGTCTCAATGC |
| TaqMan probe | 6FAM-CTCCTTCTTCTGCGAGCTCTGGACCTC-TAMRA |
| β_2 -adrenergic receptor (accession no. NM_174231) | |
| Forward | CAGCTCCAGAAGATCGACAAATC |
| Reverse | CTGCTCCACTTGACTGACGTTT |
| TaqMan probe | 6FAM-AGGGCCGCTTCCATGCCC-TAMRA |
| Calpastatin (accession no. X67333) | |
| Forward | CCCTGGATCAACTTTCTGACAGT |
| Reverse | TGACTTTATCCTCTACAGGTTTATTCTCA |
| TaqMan probe | 6FAM-TCGGGCAAAGACAGCCTGATCCA-TAMRA |
| MHC I (accession no. AB059400) | |
| Forward | CCCATTCTCCCTGATCCACTAC |
| Reverse | TTGAGCGGGTCTTTGTTTTTCT |
| TaqMan probe | 6FAM-CCGGCACGGTGGACTACAACATCATAG-TAMRA |
| MHC IIA (accession no. AB059398) | |
| Forward | CCCCGCCCCACATCTT |
| Reverse | TCTCCGGTGATCAGGATTGAC |
| TaqMan probe | 6FAM-TCTCTGACAACGCCTATCAGTTCAT-TAMRA |
| MHC IIX (accession no. AB059399) | |
| Forward | GGCCCACTTCTCCCTCATTC |
| Reverse | CCGACCACCGTCTCATTCA |
| Taqman probe | 6FAM-CGGGCACTGTGGACTACAACATTACT-TAMRA |
| RPS9 (accession no. DT860044) | |
| Forward | GAGCTGGGTTTGTCGCAAAA |
| Reverse | GGTCGAGGCGGGACTTCT |
| Taqman probe | 6FAM-ATGTGACCCCGCGGAGACCCTTC-TAMRA |

Table 2.3 Effects of implanting with Revalor-S (RS) and feeding zilpaterol-HCl (ZH; fed for the final 30 d on feed plus a 3 d withdrawal) on performance during the final 91 d on feed by finishing steers

| Item | No ZH | | ZH ¹ | | SEM ³ | P-value | | |
|------------------------------------|-------|-----------------|-----------------|-----------------|------------------|---------|-------|---------|
| | No RS | RS ² | NO RS | RS ² | | RS | ZH | RS x ZH |
| Initial BW, kg | 426.1 | 426.5 | 426.0 | 426.2 | 6.37 | 0.72 | 0.84 | 0.91 |
| Final BW, kg | 589.6 | 603.6 | 600.2 | 614.0 | 9.23 | <0.01 | <0.01 | 0.96 |
| ADG ⁴ , kg | 1.81 | 1.96 | 1.92 | 2.07 | 0.083 | <0.01 | <0.01 | 1.00 |
| DMI, kg/d | 10.31 | 10.54 | 10.21 | 10.44 | 0.269 | 0.02 | 0.27 | 0.99 |
| G:F ⁵ | 0.176 | 0.186 | 0.188 | 0.198 | 0.0036 | <0.01 | <0.01 | 0.63 |
| HCW, kg | 371.1 | 382.2 | 392.6 | 402.6 | 5.97 | <0.01 | <0.01 | 0.60 |
| Dressing percentage | 62.94 | 63.31 | 65.42 | 65.57 | 0.139 | 0.05 | <0.01 | 0.38 |
| LM area, cm ² | 90.1 | 94.8 | 101.7 | 107.0 | 1.28 | <0.01 | <0.01 | 0.66 |
| 12 th rib fat depth, cm | 1.52 | 1.50 | 1.40 | 1.35 | 0.033 | 0.31 | <0.01 | 0.78 |
| Marbling score ⁵ | 369.5 | 353.4 | 339.9 | 323.5 | 6.53 | <0.01 | <0.01 | 0.96 |
| KPH, % | 2.16 | 2.09 | 2.02 | 1.90 | 0.066 | <0.01 | <0.01 | 0.15 |

¹Zilpaterol-HCl (ZH) inclusion in the diet (8.38 mg/kg of feed on a DM basis) for the last 30 days of feed with a 3-d withdrawal.

²Revalor-S (RS) implantation (120 mg TBA and 24 mg E₂) 91 d before harvest.

³Pooled SEM of simple-effect means n = 6 pens/treatment with 90 to 100 steers/pen initially and 89 to 100 steers/pen at harvest.

⁴A 4% shrink was applied to initial and final live weights; dead or removed animals did not contribute to initial or final BW.

⁵300 = Slight; 400 = Small; 500 = Modest.

CHAPTER 3 - Effects of trenbolone acetate (TBA), estradiol-17 β (E₂), and combined TBA/E₂ implants on the levels of myosin heavy chain mRNA in skeletal muscle of feedlot steers.

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Abstract

Twenty crossbred yearling steers (421 kg) were used to evaluate the effects of implanting with trenbolone acetate (TBA), estradiol-17 β (E₂), and the combination of TBA and E₂ on finishing steer performance and myosin heavy chain (MHC) mRNA concentrations. Animals were blocked by BW, and within each block, assigned to 4 treatments. Animals were housed and fed in individual pens. Treatments consisted of: 1) no implant (CON); or implantation with 2) TBA (120 mg), 3) E₂ (25.7 mg), and 4) combined implant (120 mg TBA and 24.0 mg E₂; TBA/E₂). There were 5 animals per treatment. All animals were weighed weekly, and muscle biopsy samples were taken from the LM of each steer on d 0 (prior to implantation), d 7, d 14, and d 28. Total RNA was isolated from each sample and real-time PCR was used to measure the quantity of the 3 isoforms of bovine MHC mRNA. Total BW gain from the 28-d period was adjusted to d 0 by use of covariant analysis, and there was a tendency ($P = 0.09$) for implant to increase weight gain over CON steers. Analysis of the gene expression of MHC showed that neither implant nor day had a significant effect on the expression of type-I or -IIX MHC mRNA. There was also no treatment effect on MHC-IIA, but increasing days on feed increased ($P = 0.05$) the expression of MHC-IIA mRNA. Results of this study indicate that implanting with TBA, E₂, or both increased BW gain of finishing steers without significantly affecting the concentration of type-I, -IIA, or -IIX MHC mRNA. Increasing days on feed increased the levels of MHC-IIA in bovine skeletal muscle. We conclude that administration of steroidal implants has no effect on the proportion of the 3 different MHC mRNA isoforms in bovine skeletal muscle.

Introduction

It is well established that administration of anabolic steroid hormones improves the rate of lean growth in beef animals (Bartle et al., 1992; Hayden et al., 1992; Samber et al., 1996; Birkelo et al., 2003; Platter et al., 2003). In 1992, the FDA approved the use of implants containing the combination of androgenic and estrogenic steroids. Beef steers implanted with the combination of trenbolone acetate (TBA) and estradiol-17 β (E₂) have increased ADG, G:F, and LM area, without changes in DMI (Johnson et al., 1996; Pampusch et al., 2003). Though the improved performance associated with the combined implant is well documented and accepted, the processes by which these results are obtained are not so well understood. Satellite cells play a key role in postnatal skeletal muscle growth by contributing necessary DNA for tissue fiber hypertrophy (Allen et al., 1979). Hormone implants are thought to elicit biological responses in skeletal muscle by increasing protein synthesis and decreasing protein degradation. It is thought that protein synthesis and subsequent skeletal muscle hypertrophy of implanted cattle may be due in part to anabolic steroid hormones affecting the activation of quiescent satellite cells (Johnson 1998). One way to evaluate changes in protein synthesis and degradation is through measuring the gene expression of major proteins that are synthesized in skeletal muscle. Myosin is an important part of the contractile machinery in bovine skeletal muscle, and it exists in 3 isoforms (type-I, -IIA, and -IIX) which contribute to the functional diversity of muscle fibers (Pette and Staron, 2000). In the bovine species the MHC-IIB isoform has not been detected in skeletal muscle (Chikuni et al., 2004; Toniolo et al., 2005). Muscle hypertrophy results in an increase in the relative diameter of muscle fibers. The faster twitch, more glycolytic myosin isoforms have larger fiber diameter and exist in higher frequencies in muscle that experience the greatest amount of muscle hypertrophy in growing animals (Gunawan et al., 2007a). Transitions of fiber type in muscle can be used to show that gene expression of a fully differentiated postmitotic cell can be altered by hormonal factors and age (Pette and Staron, 2000). Hormonal differences, especially testosterone, may contribute to the gender differences in specific fiber type sizes that ultimately affect the relative concentration of MHC isoforms (Staron et al., 2000). Differences in fiber type have also been associated with meat quality in pigs (Sosnicki et al., 1987; Chang et al., 2003). Changes in the fiber type of MHC indicate potential alterations in protein synthesis, degradation, or both. Currently, there is little information published on the in

vivo effects of TBA and E₂ on skeletal muscle fiber type of MHC mRNA in finishing steers. The purpose of this study was to investigate the effects of TBA and E₂ on skeletal muscle MHC mRNA abundance.

Materials and Methods

Animals.

All experimental procedures were approved by the Kansas State University Institutional Animal Care and Use Committee, and performed at the Kansas State University Beef Cattle Research Center. Twenty yearling crossbred steers with an average initial BW of 421 ± 3.6 kg were blocked by BW and randomly assigned within block, in a 2 x 2 factorial arrangement, to 4 treatments: 1) no implant (CON), 2) implant with 25.7 mg estradiol-17 β (Compudose, Vetlife Inc., West Des Moines, IA; E₂); 3) implant with 120 mg of trenbolone acetate (Finaplix, Intervet Inc., Millsboro, DE; TBA), 4) implant with 120 mg TBA and 24 mg E₂ (Revalor-S, Intervet Inc., Millsboro, DE; TBA/E₂). On 21 d before implant, all animals were placed in individual feeding stalls and transitioned onto a 93% concentrate diet and offered feed for ad libitum intake throughout the study (Table 3.1). All steers were consuming the 93% concentrate diet on d 0. A single implant was administered subcutaneously in the right ear on d 0, after biopsy procedures, to appropriate treatment groups with the CON group receiving no implant. The live BW of each steer was measured on d 0, d 7, d 14, and d 28, prior to biopsy procedure, for analysis of performance.

Longissimus Muscle Biopsy.

Biopsy samples of the LM (1.0 g) were collected between the 10th and 13th rib from all steers before implant on d 0, and subsequently at d 7, 14, and 28. Briefly, steers were restrained in a hydraulic squeeze chute, hair was removed from the biopsy site and a local anesthetic (lidocaine HCl; 20 mg/mL; 8 mL per biopsy site) was administered. Biopsy site was cleaned with 70% ethanol and sterile surgical gauze. A 1-cm incision was made with a sterile scalpel. Tissue was collected from the LM utilizing a sterile Bergstrom biopsy needle. The incision site was closed with veterinary tissue glue. A topical antibiotic spray was applied to the incision site and then covered with a spray-on aluminum bandage. All steers were monitored for swelling 24 and 48 h after biopsy. Samples were obtained on alternate sides of the animal relative to the

previous collection, and the third and fourth biopsy samples were obtained 5 cm anterior to previous biopsy locations.

Sample Preparation and RNA Isolation.

Muscle biopsy samples from each steer were placed in 10 mL of a 5 M guanidine thiocyanate, 50 mM Tris-HCl, 25 mM EDTA, 0.5% lauryl sarcosine, and 1% β -mercaptoethanol solution in polypropylene tubes. Samples were homogenized, rapidly frozen in liquid N₂, and stored at -80°C for subsequent RNA isolation (Chomczynski and Sacchi, 1987). The RNA was mixed with one half volume LiCl precipitation solution (7.5 M LiCl, Ambion, Austin, TX) and incubated at -20°C for 30 min. The solution was then centrifuged at high speed in a microcentrifuge for 15 min. The resulting pellet was washed with 70% ethanol, dried, and resuspended in water. Samples were treated with DNase (DNA-Free, Ambion, Austin, TX) to remove any contaminating genomic DNA. The RNA concentration was determined by absorbance at 260 nm. The integrity of the RNA was determined by electrophoresis of total RNA through a 1% agarose-formaldehyde gel followed by ethidium bromide staining to allow visualization of 28 and 18S ribosomal RNA (rRNA). The RNA was then reverse transcribed to produce first-strand complementary DNA (cDNA) as described below.

Real-Time Quantitative PCR.

Real-time quantitative PCR was used to measure MHC-I, MHC-IIA, and MHC-IIX mRNA gene expression relative to the quantity of ribosomal 18S (18S) in total RNA isolated from muscle tissue. Measurements of the relative quantity of cDNA was performed by using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 900 nM of the appropriate forward and reverse primers, 200 nM of the appropriate TaqMan detection probe, and 1 μ L of the cDNA mixture. The bovine-specific MHC-I, MHC-IIA, and MHC-IIX forward and reverse primers and TaqMan detection probes (Table 3.2) were synthesized by using published GenBank sequences. Commercially available eukaryotic 18S rRNA primers and probes were used as an endogenous control (Applied Biosystems, Foster City, CA; Genbank Accession No. X03205). The ABI Prism 7000 detection system (Applied Biosystems, Foster City, CA) was used to perform the assay by using the recommended thermal cycling variables from the manufacturer (50 cycles of 15 s at 95°C and 1 min at 60°C). The 18S rRNA endogenous control was used to normalize the expression of MHC-I, MHC-IIA, and MHC-IIB isoforms.

Statistical Analysis.

Data were analyzed as a 2 x 2 factorial arrangement of treatments in a randomized complete block design with PROC MIXED procedure in SAS for Windows release 9.1 (SAS Inst. Inc., Cary, NC). Steers were blocked by weight, and animal served as experimental unit for all statistical analysis. Main effect and interaction means were separated ($P < 0.05$) with the LSD procedure of SAS.

Results and Discussion

Performance.

The performance data is shown in Table 2.3. The average initial BW (421 ± 3.6 kg) of all treatment groups did not differ on d 0. The final BW of the implanted groups tended ($P < 0.10$) to be greater than CON steers. There was a treatment by day interaction ($P < 0.05$) as BW of steers implanted with only TBA was greater than CON steers by d 14 ($P < 0.05$), and on d 28 all implanted groups were heavier than CON steers ($P < 0.05$). Dry matter intake differences among treatment groups were numerically large enough to be biologically important, however, these differences were not significant. These results support similar studies with steers administered TBA and E_2 , which show that implants containing TBA and E_2 improve ADG, G:F and the deposition of carcass lean tissue (Bartle et al., 1992; Johnson et al., 1996). Another study analyzed the effects of TBA, E_2 , and TBA/ E_2 in yearling steers and found the deposition of skeletal muscle protein was markedly increased within 40 d post-implantation (Hayden et al., 1992). Our performance results support these findings, but there is a lack of strong statistical support, likely due in part to the small number of animals per treatment used in this study.

Myosin Heavy Chain Gene Expression.

In the current study, we analyzed the expressed levels of MHC isoform types I, IIA, and IIX mRNA in steer LM tissue by real-time PCR. Data for all gene expression is shown in Figures 3.1-3.6. There was no treatment, day, or treatment by day interaction on the abundance of MHC-I (slow-oxidative) in LM tissue of steers. There was no treatment or treatment by day interaction on the expression of MHC-IIA, however there was an increase ($P = 0.05$) in MHC-IIA as days on feed increased. No treatment, day, or treatment by day interaction was observed for the expression of MHC-IIX. These results indicate that neither TBA, E_2 , nor the combination of

TBA+E₂ affect the mRNA expression of MHC-I, -IIA, or -IIX early in the feeding period following implantation. However as days on feed increases, there was an increase in the amount of MHC-IIA mRNA that is presumably available for the subsequent translation of the MHC-IIA protein.

To date there have been no studies that have analyzed the effects of anabolic steroid implants on the mRNA abundance of MHC isoforms in the LM of finishing steers. There have been attempts to determine the effect of anabolic steroids on MHC protein fiber types and size through histochemical analysis procedures. Steers administered a Synovex-S implant (20 mg 17- β estradiol benzoate and 200 mg progesterone) had no effect on the fiber type distribution or area in the LM (Ono et al., 1996). Another study showed that implant treatments with Revalor-S, Ralgro (zeranol), or Synovex-S had no effect on either percentage of fiber type or the cross sectional area of slow-twitch-oxidative, fast-twitch-oxidative-glycolytic, or fast twitch-glycolytic fibers in the LM of steers (Fritsche et al., 2000). Gonzalez et al. (2007) found that TBA alone had no effect on the percentage, computed cross sectional area, or diameter of type II muscle fibers, and no effect on the percentage of type I muscle fibers in the LM of cull beef cows. However, TBA did increase cross sectional area and diameter of type I muscle fibers in the LM (Gonzalez et al., 2007). These data are similar to the results of our studies that anabolic steroids appear to have little effect on MHC mRNA levels. It is important to note that these studies measured the distribution of fiber types found in muscle by means of histochemical and immuno-histochemical analysis, whereas our study analyzed the mRNA abundance of the MHC isoforms by real-time PCR in skeletal muscle samples obtained by serial biopsy.

Gene Expression Overview.

Myosin heavy chain isoforms are unique types of protein that research has worked to better understand and classify. There have been 4 main isoforms of MHC mRNA detected in porcine skeletal muscle, however only 3 of these isoforms have been detected in bovine skeletal tissue. In general, type IIB MHC mRNA is not expressed in skeletal muscle of cattle (Chikuni et al., 2004; Gonzalez et al., 2007), with the exception being the extraocular muscles which are the only skeletal muscles known to express MHC-IIB mRNA in cattle (Toniolo et al., 2005). Therefore, we did not analyze the LM tissue for the expression of MHC-IIB mRNA.

Muscle fiber types are classified according to functional (slow-twitch vs. fast-twitch) and metabolic (glycolytic vs. oxidative) properties as well as radial growth. Previous studies have

struggled with accurate correlations between myosin composition and fiber type distribution of skeletal muscle. It appears that MHC isoforms are the most appropriate markers for fiber type delineation (Pette and Staron, 2000). The 2 factors that have complicated this issue are: 1) a continuum of fiber types exist and multiple myosin heavy chains can coexist in the same fiber type (Bee et al., 1999), and 2) the molecular identification of fiber types based on MHC expression is more precise and reliable than immuno-histochemical staining (Kim et al., 2008). With regard to the extraocular muscles in bovine, there is evidence that MHC-IIB is expressed at the protein level in those muscles where the corresponding mRNA is present (Toniolo et al., 2005). Also, the expression of MHC genes was highly correlated with their relative amount of the corresponding protein contained within porcine skeletal muscle (Gunawan et al., 2007b). Therefore, gene expression of MHC mRNA has been determined to be an appropriate and accurate indicator of the amount and type of protein that is translated in skeletal muscle, and was the method chosen for the current study.

It is well documented that another form of growth promotants known as β -adrenergic agonists (β -AA) can alter the expression of MHC. Administration of β -AA causes a shift in the amount of isoform expressed from slower, oxidative to faster, glycolytic fiber types, as well as dramatically increasing the size of the type IIB fibers (Beermann et al., 1987; Zeman et al., 1988; Polla et al., 2001). Both steroid hormones and β -AA have been shown to increase the accumulation of lean tissue in skeletal muscle of cattle. Due to the similar nature of response on skeletal muscle deposition, we expected to observe similar patterns of change in the gene expression of MHC. Interestingly, we did not observe similar results, but rather no effects were observed due to treatment of steroid hormones. Steroid hormones and β -AA elicit a similar growth response in regard to lean tissue accumulation, but these observations imply that responses occur through different pathways.

In a previous study with rats administered a high therapeutic dose of nortestosterone over a 25-d period, there were no modifications in muscle phenotype (Noirez and Ferry, 2000). These results support our observation of the lack of effect from the treatments containing trenbolone. It is important to note that different species have variation in type and amount of myosin genes expressed, as well as their physiological response to steroid hormones.

There are various factors that can affect MHC fiber type transitions, one of which is the effect of the mechanical load exerted on the muscle (Pette and Staron, 2000). This could be a

possible factor in the change in fiber type observed in our study. The reduction in pen size where the animals were housed during our study could have affected the physical activity and mechanical load on skeletal muscle; however, this would have a greater impact on skeletal muscles of the limbs, rather than a trunk muscle like the LM. Therefore, the impact of physical activity and skeletal muscle load on the expression of MHC of the LM muscles would likely be minimal, if any at all. Furthermore, all animals were acclimated to the individual pens 21 d before starting the study, thus reducing the effect of changes in physical activity.

Another factor that is related to variations in MHC isoforms expressed in bovine skeletal muscle is meat quality. It has been shown that MHC isoform composition contributes to the variations observed in postmortem energy metabolism, susceptibility to adverse pH, temperature effects, and ultimately meat quality in porcine muscle (Bowker et al., 2004). Steers that received aggressive implant protocols during the finishing stages of development had carcasses that were lower in both marbling and tenderness than those of non-implanted steers, and increasing the amount of implants a steer receives over its lifetime further reduces marbling and tenderness (Platter et al., 2003). Integrating these results with the findings of the current study would imply that the gene expression of myosin may not be related to the carcass characteristics of tenderness and marbling in beef steers, although we only analyzed changes in MHC over the first 28 d following administration of the implants.

General Conclusion

In growing beef steers there are various factors that affect protein synthesis/degradation and ultimately lean tissue accumulation. Steroid hormones are compounds that accelerate this growth process by pathways that are still not fully understood. An alteration in the gene expression of MHC mRNA is one indicator of changes in protein synthesis and degradation. It is evident that implants containing TBA and E₂ do not alter the mRNA gene expression of MHC isoforms during the first part of the feeding period of growing beef steers; however increasing the days on feed did increase the abundance of MHC-IIA.

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Figures and Tables

Figure 3.1. Myosin heavy chain type I mRNA abundance in bovine LM tissue in yearling steers collected at 4 different biopsy days. Treatments included: 1) no implant (CON); or implant with 2) 25.7 mg estradiol-17 β (E₂); 3) 120 mg trenbolone acetate (TBA); and 4) combined implant of 120 mg trenbolone acetate and 24 mg estradiol-17 β ; (TBA/E₂).

Biopsies were taken from 5 steers in each of the 4 treatment groups. Biopsy samples were collected on d 0, 7, 14, and 28 after administration with steroidal implant. Total RNA was isolated from skeletal muscle tissue and relative MHC-I gene expression was determined using real-time quantitative-PCR. There were no effects due to treatments on the expression of MHC-I. Error bars are SEM.

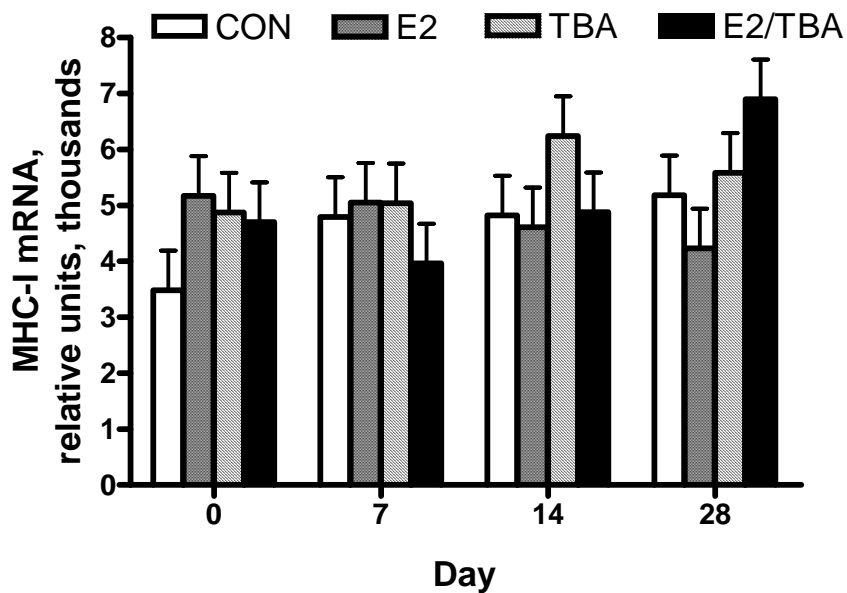


Figure 3.2. Myosin heavy chain type IIA mRNA abundance in bovine LM tissue in yearling steers collected at 4 different biopsy days. Treatments included: 1) no implant (CON); or implant with 2) 25.7 mg estradiol-17 β (E₂); 3) 120 mg trenbolone acetate (TBA); and 4) combined implant of 120 mg trenbolone acetate and 24 mg estradiol-17 β ; (TBA/E₂). Biopsies were taken from 5 steers in each of the 4 treatment groups. Biopsy samples were collected on d 0, 7, 14, and 28 after administration with steroidal implant. Total RNA was isolated from skeletal muscle tissue and relative MHC-IIA gene expression was determined using real-time quantitative-PCR. There were no effects due to treatments on the expression of MHC-IIA. Bars represent standard error within treatments.

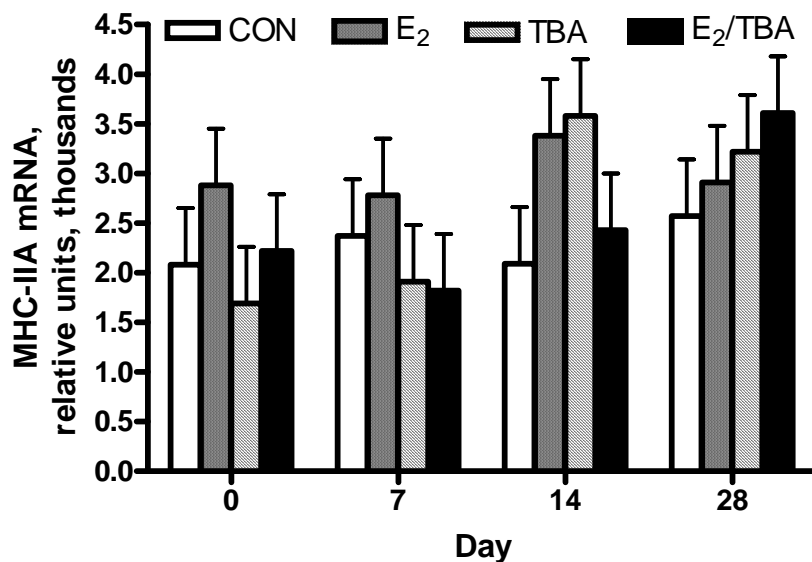


Figure 3.3. Myosin heavy chain type IIX mRNA abundance in bovine LM tissue in yearling steers collected at 4 different biopsy days. Treatments included: 1) no implant (CON); or implant with 2) 25.7 mg estradiol-17 β (E₂); 3) 120 mg trenbolone acetate (TBA); and 4) combined implant of 120 mg trenbolone acetate and 24 mg estradiol-17 β ; (TBA/E₂). Biopsies were taken from 5 steers in each of the 4 treatment groups. Biopsy samples were collected on d 0, 7, 14, and 28 after administration with steroidal implant. Total RNA was isolated from skeletal muscle tissue and relative MHC-IIX gene expression was determined using real-time quantitative-PCR. There were no effects due to treatments on the expression of MHC-IIX. Error bars are SEM.

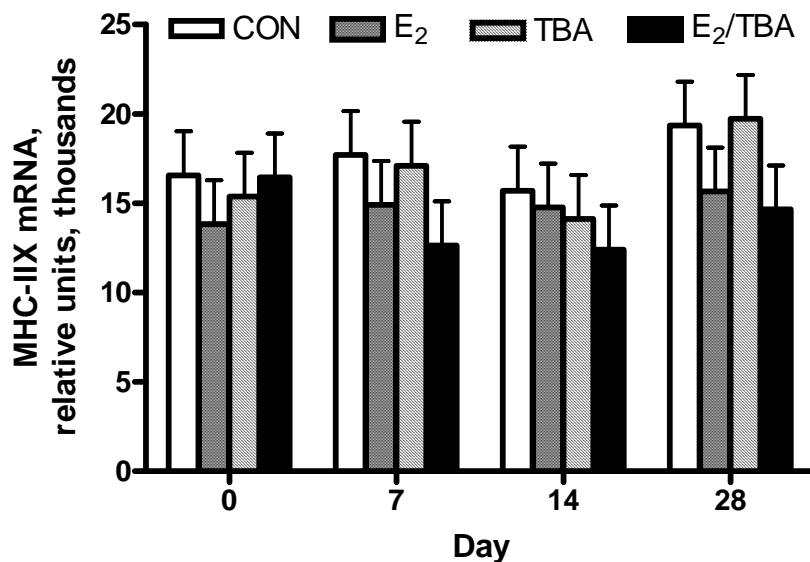


Figure 3.4. Myosin heavy chain type I mRNA abundance in bovine LM tissue in yearling steers collected on 4 different biopsy days. Biopsy samples were collected on d 0, 7, 14, and 28 on feed from 20 individual steers. Total RNA was isolated from skeletal muscle tissue and relative MHC-I gene expression was determined using real-time quantitative-PCR. There was no effect on the expression of MHC-I with increasing days on feed. Error bars are SEM.

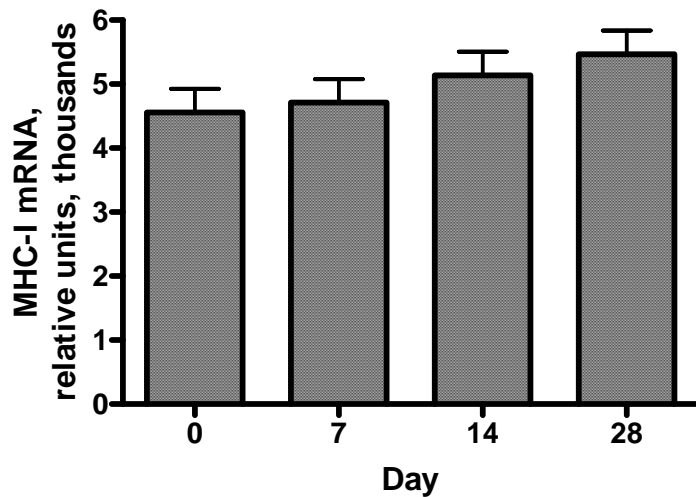


Figure 3.5. Myosin heavy chain type IIA mRNA abundance in bovine LM tissue in yearling steers collected on 4 different biopsy days. Biopsy samples were collected on d 0, 7, 14, and 28 on feed from 20 individual steers. Total RNA was isolated from skeletal muscle tissue and relative MHC-IIA gene expression was determined using real-time quantitative-PCR. There was a significant ($P < 0.05$) increase in the expression of MHC-IIA with increasing days on feed. Error bars are SEM.

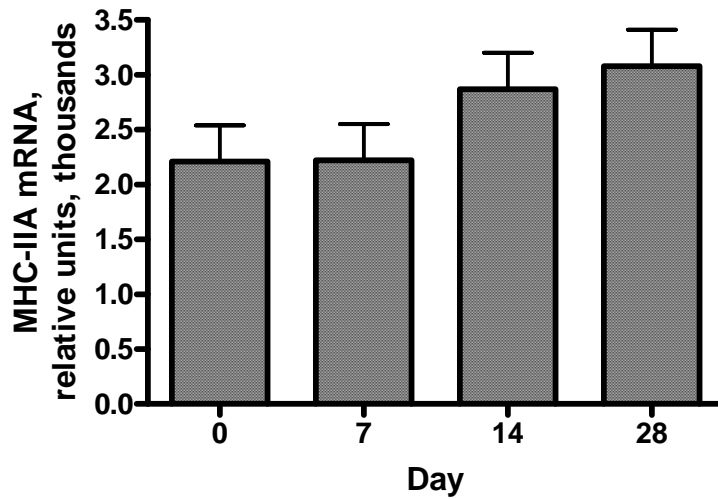


Figure 3.6. Myosin heavy chain type IIX mRNA abundance in bovine LM tissue in yearling steers collected on 4 different biopsy days. Biopsy samples were collected on d 0, 7, 14, and 28 on feed from 20 individual steers. Total RNA was isolated from skeletal muscle tissue and relative MHC-IIX gene expression was determined using real-time quantitative-PCR. There was no effect on the expression of MHC-IIX with increasing days on feed. Error bars are SEM.

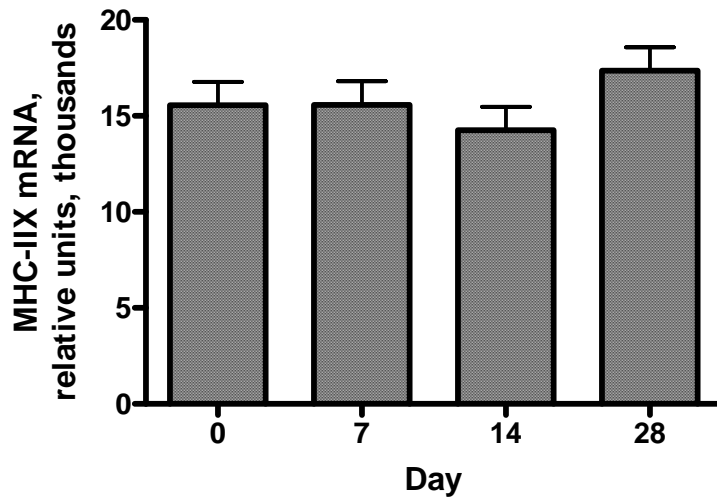


Table 3.1. Composition and analyzed nutrient content of the finishing diet

| Item | % of DM |
|------------------------------------|---------|
| Corn, steam-flaked | 78.09 |
| Alfalfa hay, ground | 11.96 |
| Corn steep liquor | 4.31 |
| Supplement ¹ | 3.46 |
| Rumensin/Tylan premix ² | 2.18 |
| DM, % | 79.71 |
| CP, % of DM | 13.56 |
| NDF, % of DM | 14.47 |
| NEm, Mcal/lb | 2.09 |
| NEg, Mcal/lb | 1.42 |

¹The supplement contained (DM basis): 45.29% limestone, 35.20% urea, 8.69% salt, 7.28% potassium, 1.45% vitamin E (20,000 IU/g), 1.28% trace mineral premix (3.67 mg/kg Co, 291.15 mg/kg Cu, 18.21 mg/kg iodineum, 1747.83 mg/kg Mn, 7.28 mg/kg Se, 1745.90 mg/kg Zn), 0.60% sodium selenium, and 0.21% vitamin A (30,000 IU/g)

²Rumensin/Tylan premix contained (DM basis): 97.30% dry rolled corn, 1.50% soybean oil, 0.75% Rumensin (1480 mg/kg; Elanco Animal Health, Indianapolis, IN), and 0.45% Tylan (445 mg/kg; Elanco Animal Health, Indianapolis, IN)

Table 3.2. Sequences for bovine-specific PCR primers and Taqman probes of type I, IIA, and IIX myosin heavy chain (MHC) mRNA.

| Item | Sequence (5' to 3') |
|----------------------------------|--|
| MHC I (accession no. AB059400) | |
| Forward | CCCACTTCTCCCTGATCCACTAC |
| Reverse | TTGAGCGGGTCTTTGTTTTTCT |
| TaqMan probe | 6FAM-CCGGCACGGTGGACTACAACATCATAG-TAMRA |
| MHC IIA (accession no. AB059398) | |
| Forward | CCCCGCCCCACATCTT |
| Reverse | TCTCCGGTGATCAGGATTGAC |
| TaqMan probe | 6FAM-TCTCTGACAACGCCTATCAGTTCAT-TAMRA |
| MHC IIX (accession no. AB059399) | |
| Forward | GGCCCACTTCTCCCTCATTC |
| Reverse | CCGACCACCGTCTCATTCA |
| Taqman probe | 6FAM-CGGGCACTGTGGACTACAACATTACT-TAMRA |

Table 3.3. Cumulative performance by treatment

| Item | Implant dose (E ₂ :TBA), mg | | | | SEM ¹ |
|---------------------|--|--------|-------|--------|------------------|
| | 0:0 | 25.7:0 | 0:120 | 24:120 | |
| No. of observations | 4 | 5 | 5 | 5 | -- |
| Initial BW, kg | 419.1 | 417.8 | 421.8 | 421.8 | 2.94 |
| 0 to 14 d | | | | | |
| BW, kg | 438.6 | 444.1 | 452.7 | 444.5 | 4.08 |
| ADG, kg | 1.39 | 1.89 | 2.21 | 1.62 | 0.232 |
| DMI, kg | 7.96 | 8.77 | 9.19 | 8.13 | 0.416 |
| 15 to 28 d | | | | | |
| BW, kg | 450.0 | 464.5 | 467.7 | 469.9 | 5.53 |
| ADG, kg | 0.81 | 1.48 | 1.07 | 1.81 | 0.271 |
| DMI, kg | 7.33 | 8.95 | 9.03 | 8.39 | 0.486 |
| 0 to 28 d | | | | | |
| BW gained, kg | 30.9 | 46.7 | 45.9 | 48.1 | 5.53 |
| ADG, kg | 1.10 | 1.67 | 1.64 | 1.72 | 0.197 |
| DMI, kg | 7.65 | 8.86 | 9.11 | 8.26 | 0.411 |
| G/F | 0.144 | 0.188 | 0.180 | 0.208 | 0.015 |

¹Calculated using 5 observations per treatment